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ANTI-(ACETYLCHOLINE RECEPTOR) ANTIBODIES IN MYASTHENIA GRAVIS

Submitted by HELEN SYLVIA LOTWICK

For the degree of Ph.D. of the

University of Bath

1985

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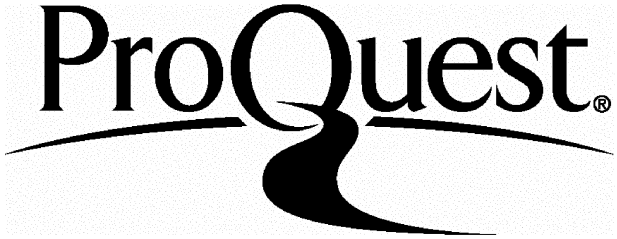

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SUMMARY

Levels of anti-(AChR) antibodies were determined in serial serum samples from 14 myasthenic patients over a period of several months, using detergent-solubilized muscle extracts of junctional rat AChR, extra-junctional rat AChR and human adult AChR as antigens. Anti-(AChR) antibody titres obtained using human adult AChR were always higher than those obtained using extra-junctional rat AChR, which were, in turn, always higher than those obtained using junctional rat AChR. The ratios of antibody titres obtained by using the different antigens varied between patients, but were constant for an individual over the period of study. Complementary evidence for the same phenomenon was obtained by other experiments in which an excess of each serum was used to precipitate limited amounts of AChR from muscle extracts. The results obtained by combining myasthenic sera argue against the suggestion that incomplete precipitation of receptor by certain sera is caused by the absence of particular antibody sub-populations. An alternative explanation, that sera precipitating low amounts of AChR contain toxin-releasing antibodies, is supported by direct measurements of antibody-mediated toxin loss.

The hypothesis that embryonic AChR may constitute the autoimmunogen in MG was investigated by comparing the interaction of human foetal AChR with α-BGT and anti-(AChR) antibodies against that established for adult human AChR. Tissue sections and teased muscle fibres from human adult and foetal muscle were compared immunohistochemically. Detergent extracts of adult and foetal AChRs were compared in their interaction with radiolabelled α-BGT by

kinetic measurements involving determination of association, dissociation and equilibrium binding constants. AChR was isolated and partially purified from human adult and foetal muscle, and their binding to anti-(AChR) antibodies in myasthenic sera and IgG were compared. No significant difference was observed between the binding characteristics of the two receptor types, indicating the absence (at least in 14 - 22 week old foetuses) of ligand binding or antigenic sites unique to foetal AChR.

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ABBREVIATIONS

Ab	Antibody
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
J-AChR	Junctional AChR
EJ-AChR	Extra-junctional AChR
α-BGT	α-bungarotoxin
[^{125}I]α-BGT	[^{125}I]α-bungarotoxin
FITC-α-BGT	Fluoroisothiocyanate conjugated to α-BGT
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribose Nucleic Acid
EAMG	Experimental Autoimmune Myasthenia Gravis
EDTA	Ethylene diamine tetraacetic acid
EPP	End-plate potential
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IgG	Immunoglobulin G
MEPP	Mini end-plate potential
MG	Myasthenia Gravis
PAP	Horseradish peroxidase anti-(peroxidase) complex
PMSF	Phenylmethylsulphonyl fluoride
PPO	2,5-diphenyloxazole
RIA	Radioimmunoassay
mRNA	Messenger Ribose Nucleic Acid
Triton X-100	Isooctyl phenoxypolyethoxyethanol

CONTENTS

INTRODUCTION

"Nevertheless, those labouring with a want of spirits, will use these spirits for local motions as well they can; in the morning they are able to walk firmly, to fling their arms about hither and thither or to take up any heavy thing; before noon the stock of spirits being spent, which has flowed into the muscles, they are scarcely able to move hand nor foot. At this time I have under my charge a prudent and honest woman who for many years has been subject to this form of spurious palsy, not only in her members, but also in her tongue. She can for some time speak hastily or eagerly, she is not able to speak a word, but becomes suddenly as mute as a fish, nor can she recover the use of her voice for an hour or two."

Thomas Willis, 1672

The human disease myasthenia gravis (MG), the symptoms of which are vividly described above, was first documented over three hundred years ago. Since then, and especially over the last twenty years, a great volume of information has been recorded concerning the aetiology, pathogenesis and treatment of the disease (for comprehensive reviews see Lindstrom, 1979; Vincent, 1980; Vincent & Newsom-Davis, 1982a). Clinically, MG is characterized by weakness and rapid fatiguability of skeletal muscle. The disorder may be selective for a particular muscle group (eg. ocular, bulbar, limb, respiratory etc.) or generalized. It is now generally accepted that the basic defect in MG is a decrease in the number of functional acetylcholine receptors (AChRs) at the post-synaptic membrane of the neuromuscular junction, resulting from an antibody-mediated

response. Elucidation of the mechanisms involved in the pathogenesis of MG has in many ways paralleled the understanding of muscle and nerve physiology; the latter will therefore be dealt with first, before progressing to a detailed discussion of the pathology of the disease.

The organization of the neuromuscular junction

Until the latter part of the nineteenth century the structural organization of the nervous system was supposed to be a continuous network, whereby communication between cells was a continuation of the process by which nerve impulses were conducted within the boundaries of each individual cell. Ramon y Cajal (1888) challenged this theory, suggesting instead that nerve cells, although in close contact with each other, are discrete units, each enclosing its own cytoplasmic contents. Sherrington (1925) confirmed this hypothesis by electrophysiological studies, and coined the term 'synapse' to describe the specialized contacts existing between cells.

The discontinuity of the nervous system implied the presence of an additional communication system between cells; this led to the idea that a chemical messenger might be present at the synapse (Eliott, 1904; Dixon, 1906). The first convincing demonstration that this was so came from Loewi (1921) whose experiments on the vagal stimulation of isolated frog heart muscle led to the formulation of the neurotransmitter theory of synaptic transmission. Initial attempts to show that this chemical messenger, which, in the case of frog heart, Loewi later identified as acetylcholine (ACh), was released as a result of nerve stimulation were hindered by its rapid hydrolysis; but the inclusion of eserine (an acetylcholine esterase

(AChE) inhibitor) in the perfusion fluid (Loewi & Navratil, 1926; Dale et al., 1936) then facilitated its detection. Additional experiments using curare also allowed the demonstration that ACh was released by stimulation of the motor nerve and not by the action of the muscle. Pharmacological studies led Langley (1907) to propose that the muscle contains a 'receptive substance' which receives the "special substance secreted by the end of the nerve". The nerve-muscle junction is now one of the best understood systems with respect to the organization of the elements of neurotransmission. Its function is to transfer the propagated nerve impulse from the motor nerve ending to the muscle fibre, resulting ultimately in muscle contraction. In the majority of vertebrate muscles, each muscle fibre is innervated by one nerve, and the area of contact between the two is termed the neuromuscular junction (see Figure 1).

Early studies of acetylcholine receptors were dependant on the ability of the receptor to depolarize the membrane of the post-synaptic muscle cell, the large size of which facilitates intracellular recording. Neuromuscular transmission is initiated when a nerve impulse invades the nerve terminal, which can be achieved experimentally by electrical stimulation of the nerve. This increases membrane permeability to calcium ions and their influx into the nerve terminal triggers release of ACh which diffuses into and across the synaptic cleft. The binding of ACh to AChR in the post-synaptic membrane results in a conformational change which is associated with the opening of an ion channel. The channel remains open for approximately 1 millisecond allowing sodium and potassium ions to flow through it down their electrochemical gradients. More sodium ions move in than potassium ions move out, resulting in a net influx of positive charge and depolarization of the muscle membrane

Figure 1. Schematic representation of the neuromuscular junction

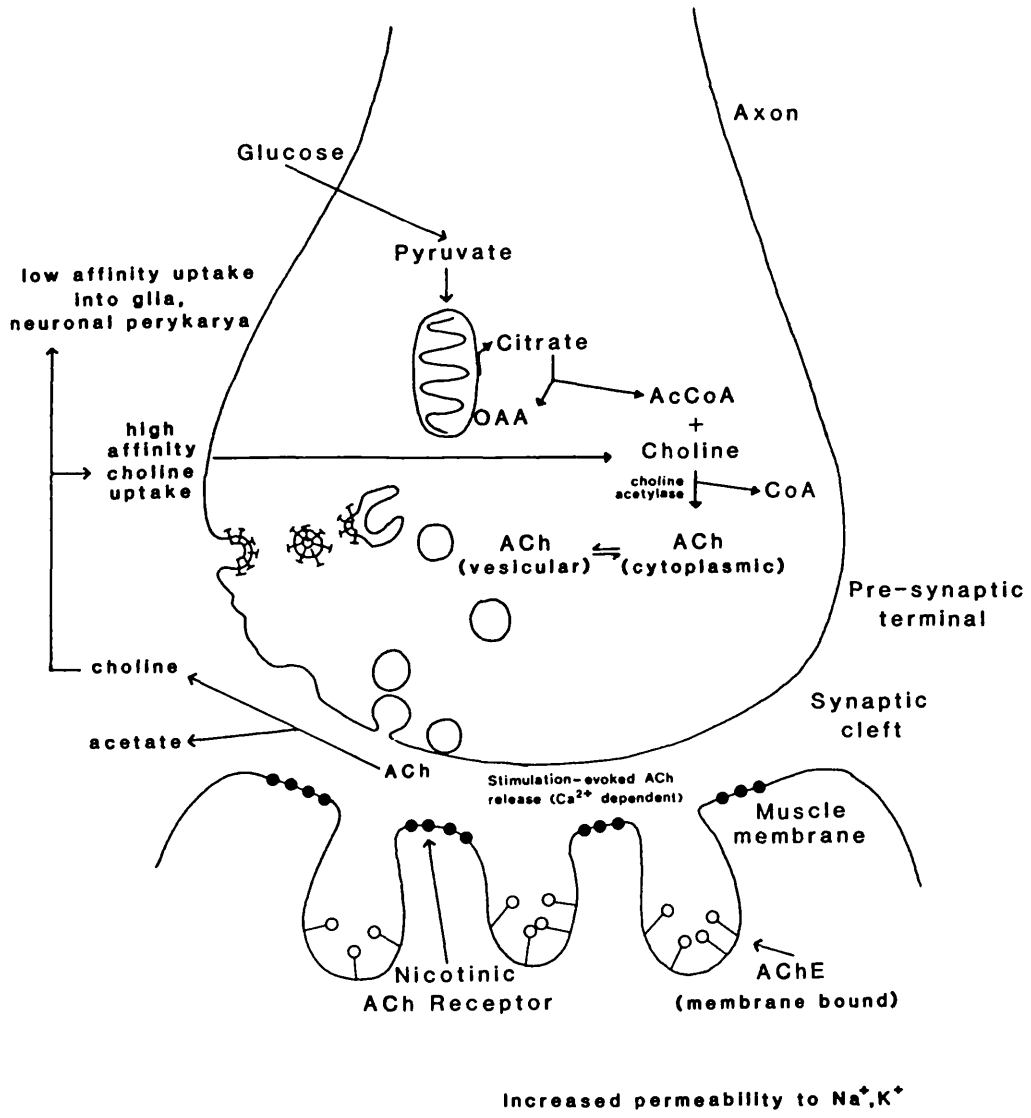
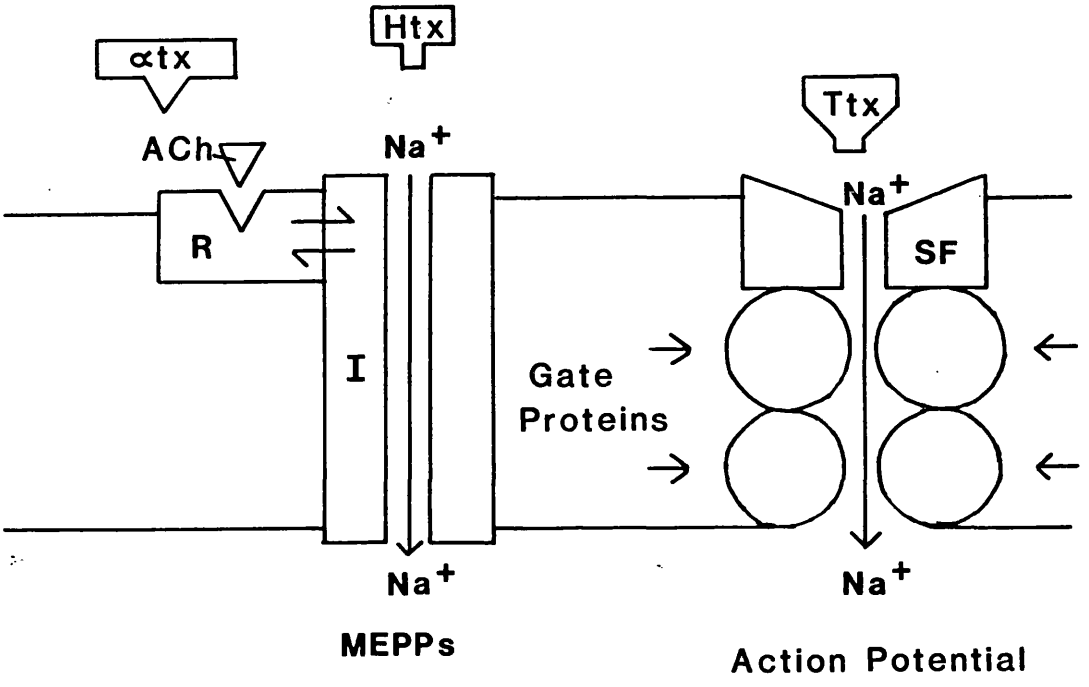


Figure 2. The initiation of an action potential at a nicotinic cholinergic synapse



Acetylcholine (ACh) interacts with the receptor (R). The interaction results in the opening of the ionophore (I) and Na^+ ions enter the cell generating mini end-plate potentials (MEPPs). The summated MEPPs change the membrane potential ($\Delta\psi$) and promote an aggregation of the intrinsic gate proteins with the selectivity filter (SF), thereby forming a functional sodium channel. The system can be blocked at 3 distinct sites by toxins: α-toxin (αtx); histrionicatoxin (Htx) and tetrodotoxin (Ttx).

producing an 'end-plate potential' (EPP). If the summation of EPPs causes depolarization to the threshold value, the muscle membrane generates an action potential which is propagated along the muscle fibre and ultimately produces its contraction. Smaller, spontaneous depolarizations, known as 'miniature end-plate potentials' (MEPPs), occur in resting muscle, at a frequency of about one per second; these result from the localized release of small quantities of ACh. Their presence has led to the formulation of the "quantal" theory of chemical transmission according to which ACh is released in discrete quanta. In humans, each quantum probably contains less than 10^4 ACh molecules, and 50 - 200 quanta are released from one nerve ending following nerve stimulation. The action of ACh is terminated by its hydrolysis by AChE, and by diffusion out of the synaptic cleft (see Figures 1 & 2).

Structural analysis of the "receptive substance" which binds ACh and initiates such contraction had to await the development of suitable techniques. Two factors which significantly and radically advanced the characterization of the AChR were, firstly, the discovery of an abundant source of the receptor in the electric organ of the electric fishes, and, secondly, the availability of the α-neurotoxins from snake venoms which bind with high affinity and specificity to the AChR.

Electric organs of electric fish

The electric organ from electric fish consists of a regular array of stacks of large, flat syncitia called electroplaques, each of which is subject to dense cholinergic innervation and produces a change in potential in response to ACh released from the nerve terminals. Because the cells are arranged in series, summation of

the evoked potentials gives rise to a discharge. The organ is embryologically similar to that of skeletal muscle, but has no contractile elements, the non-innervated face being specialized for active transport. The two most studied electric organs are those of the freshwater eel, Electrophorus electricus and various species of the Torpedo genus - the electric ray - which frequents temperate marine waters. The area of synaptic contact in these species is 2% and 50% respectively, and the fact that all synapses respond to ACh means that the electric organ is a rich and homogenous source of AChR which has been used extensively for biochemical characterizations. AChRs isolated from both electric fish and vertebrate skeletal muscle show characteristic nicotinic pharmacology.

The α-neurotoxins

The discovery that the AChR is the target of the α-neurotoxins from elapid and hydrophobid snake venoms has undoubtedly been one of the major contributions not only to the structural characterization of the receptor, but also (as will be described later) to the clarification of the role of the receptor in MG. These α-neurotoxins were first purified by Chang & Lee (1962), who demonstrated that they produce an anti-depolarizing block of the AChR at the neuromuscular junction (Lee, 1972) in a manner similar to that of the cholinergic antagonist d-tubocurarine. The α-toxins are small compact peptides comprising 61 - 74 amino-acid residues, 4 - 5 di-sulphide bridges and a net positive charge. They have been divided into two classes (Type 1 and Type 11) on the basis of their behaviour at the neuromuscular junction.

Type 1 α-toxins have 60 - 62 amino-acid residues, produce a

reversible neuromuscular block and therefore have been exploited as ligands for affinity purification of AChR (eg. the α-toxin from Naja naja siamensis).

Type 11 α-toxins have 71 - 74 amino-acid residues and bind almost irreversibly to the AChR. Their usefulness as probes for the AChR has relied upon the finding that they may be radiolabelled to high specific activity with retention of biological activity (eg. α-bungarotoxin (α-BGT) from Bungarus multicinctus).

Isolation and characterization of AChR from electric fish

AChR has been found to be an integral membrane protein, and dissolution of the membrane is accordingly a pre-requisite to any purification procedures. Extraction of AChR with non-ionic detergent (eg. Triton X-100) results in a soluble form of receptor which retains its ability to bind α-toxins and cholinergic ligands (Changeux et al., 1970; Miledi et al., 1971). Purification by affinity chromatography, biospecific elution and concentration on DEAE-cellulose yields preparations with specific activities of 5 - 10 μmol α-BGT binding sites per gram protein. The AChR has by now been extensively characterized, and its properties have been described in reviews by many groups of workers (Karlin, 1980; Changeux, 1981; Conti-Tronconi & Raftery, 1982; 1983; Barrantes, 1983; Changeux et al., 1984). The receptor is a glycoprotein containing 4 - 7% carbohydrate. It comprises four subunits, designated α,β,γ,δ in the ratio 2:1:1:1 with apparant molecular weights (from polyacrylamide gel electrophoresis) of 40000, 50000, 60000 and 65000 respectively. The α subunit contains the binding site for ACh, the role of the other three subunits is, as yet, uncertain. The primary structures of all four polypeptide chains

have been recently elucidated following the extraction of the corresponding messenger RNA (mRNA) and its translation in cell-free systems (Mendez et al., 1980; Anderson & Blobel, 1981; Sumikawa et al., 1981) and in Xenopus oocytes (Sumikawa et al., 1981; Mishina et al., 1984). Identification of the mRNA for AChR has allowed the cloning of the complementary DNA (cDNA) which has, in turn, enabled nucleotide sequencing of the α subunit from Torpedo marmorata (Sumikawa et al., 1982a; Devillers-Thiery et al., 1983) and all four subunits from Torpedo californica (Ballivet et al., 1982; Noda et al., 1982; 1983a,b). Reconstitution of the receptor into model membranes leads to restoration of ligand and ion permeability properties (Anholt, 1981; McNamee & Ochoa, 1982). The function of AChR in reconstituted membranes can be altered by changing the lipid composition in the membrane; lipids displaying the highest affinity for the AChR are those having the largest effects on the influx properties of the receptor (Ellena et al., 1983). Such studies may shed light on the properties of the ion channel and the functional contribution of the various subunits.

The purified receptor consists of rosettes 9 nm in diameter, each with a central pit of 2 nm as revealed by electron microscope (EM) studies; this agrees well with freeze-fracture images which show particles on the synaptic face of the electroplaque membrane (Cartaud et al., 1978). EM studies of anti-(AChR) antibody binding to the post-synaptic membrane show that the AChR is a transmembrane protein exposed on both the cytoplasmic and extracellular surfaces (Tarrab-Hazdai et al., 1978; Strader et al., 1979). Initial studies used to determine the extent of exposure of individual chains to the cytoplasmic face were controversial because of the disruptive treatments used (Strader & Raftery, 1980; Wennogle & Changeux,

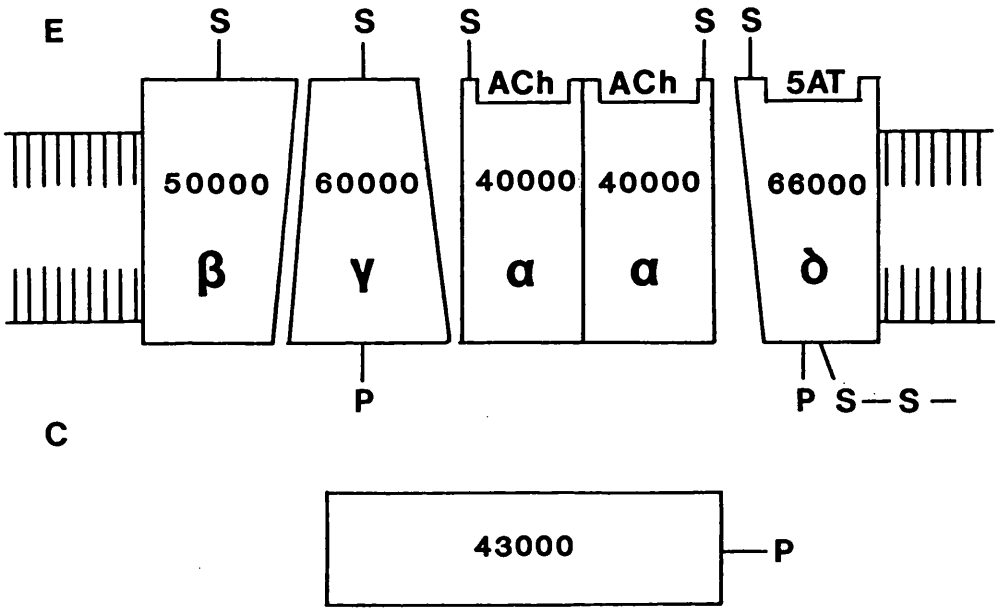
1980). Recent studies involving monoclonal antibody binding have shown that all four subunits are exposed on the cytoplasmic face (Sargent et al., 1984), while studies using lactoperoxidase catalysed iodination (Hartig & Raftery, 1977), peptide cross-linking (Witzmann & Raftery, 1978; Karlin, 1980) and proteolytic degradation (Strader & Raftery, 1980; Anderson & Blobel, 1981) indicate that all four subunits are also exposed on the extracellular surface (see Figure 3). The implications of this in the pathogenesis of MG will be discussed later.

Isolation and characterization of AChR from skeletal muscle

The isolation and characterization of AChR from vertebrate skeletal muscle has proved considerably more difficult than that for electroplaque AChR, mainly because of the very low content of AChR in the muscle membrane (Wallis et al., 1980) and also as a result of proteolysis during purification (Lindstrom et al., 1980). In normal adult innervated muscle the receptor comprises 0.002% of the total protein present (Nathanson & Hall, 1980). After denervation, however, AChRs increase 5 - 44 fold (Dolly, 1979), providing a useful, enriched source of receptors which has enabled their purification and partial characterization from a variety of sources (see TableS 20 & 22). Care must be taken in drawing conclusions from such studies, as although the receptors which appear in the muscle membrane after denervation are in many ways similar to those found at the muscle end-plate, some differences between these two receptor types are apparent (see p. 13).

Initial physicochemical studies on purified AChRs led to some controversy regarding the subunit structure of vertebrate muscle AChRs, with reports of between one and five subunits (reviewed by

Figure 3. Schematic representation of the receptor peptides in the membrane



C - Cytoplasmic medium

E - External face membrane

S - Sugar residues

ACh - Acetylcholine

5AT - 5-azidotrimethosquin binding site for non-competitive blockers

P - Site of phosphorylation

Conti-Tronconi & Raftery, 1982), which may or may not correspond to the putative α, β, γ and δ subunits of Torpedo AChR. These discrepancies have to some degree been clarified by the production of antisera and monoclonal antibodies against individual Torpedo AChR subunits which have been found to cross react with mammalian muscle AChR (Lindstrom et al., 1978b; 1979; 1980; Tzartos & Lindstrom, 1980). Furthermore, the induction of an immune response in rats to their own AChR by immunization with any of the Torpedo AChR subunits (Lindstrom et al., 1978a) suggests that mammalian muscle AChR may contain antigenic sites which are similar to those of the Torpedo AChR. Noda and co-workers (1983c) have cloned complementary DNAs for the α subunit precursor of calf skeletal muscle AChR and for a human genomic DNA containing the corresponding gene. The nucleotide sequences obtained from these studies show a large degree of sequence homology with the corresponding Torpedo cDNAs, suggesting a conservation of the AChR protein between species.

The distribution of AChR in the muscle membrane

The distribution of AChRs on muscle cells has been analysed both by recording the membrane potential during localized iontophoretic application of ACh to the cell surface, and by mapping the binding of radiolabelled α-BGT by using autoradiographic and histochemical methods (Famborough, 1979). These investigations have revealed that two classes of AChR exist in the muscle membrane:- junctional (J) and extrajunctional (EJ) receptors. Adult innervated muscle fibres contain junctional AChRs which are found at high density in the post-synaptic folds of the muscle membrane (see Figure 1), at a concentration of $2 - 6 \times 10^7$ receptors per junction

(Famborough, 1979). The enzyme AChE, which causes hydrolysis of ACh, is located along the folded membrane (Karnovsky & Roots, 1964). Extrajunctional receptors, which are barely detectable in normal adult innervated muscle, appear, following denervation, at high density over the entire surface of the muscle fibre (Axelsson & Thesleff, 1959; Miledi, 1960a; Miledi & Potter, 1971; Berg et al., 1972; Hartzell & Famborough, 1972), resulting from de novo synthesis and direct incorporation into the extrajunctional membrane (Chang & Tung, 1974). If denervated muscle is reinnervated, then a reversal of this process occurs with a reduction in the number of receptors outside the endplate until a normal adult pattern is achieved (Miledi, 1960b). Extra-synaptic receptors are also present in developing myotubes of embryonic species (Bevan & Steinbach, 1977), and, although they are similar in many respects to the extrajunctional receptors of adult denervated muscle, it is not clear whether they represent distinct molecular forms.

Comparison of junctional and extra-junctional AChRs

Extrajunctional receptors differ in several respects from junctional receptors (see Famborough 1979 for review). In particular, junctional receptors are relatively immobile, have a slower metabolic turnover time and a shorter channel open time (Katz & Miledi, 1972). Biochemical studies on affinity purified receptors have shown few physicochemical differences between the two receptor types. The rat AChRs have similar subunit compositions, as revealed by 1-dimensional peptide maps of the major (α and β) subunits (Nathanson & Hall, 1979), and are indistinguishable by gel filtration and sucrose density centrifugation (Brockes & Hall, 1975b; Froehner et al., 1977). Studies by Sumikawa and co-workers

(1982b) on AChR from chick embryonic, denervated and innervated muscle suggest that they are closely related structurally. However, some differences between junctional and extrajunctional receptors have been observed:- junctional rat AChR has a lower isoelectric point than extrajunctional AChR (Brockes & Hall, 1975b), although this was not noted for chick AChRs (Sumikawa et al., 1982b). The sensitivity of the receptors to d-tubocurarine differs:- junctional rat AChR has an apparent dissociation constant of 4.5×10^{-8} M, compared with 5.5×10^{-7}M for extrajunctional receptors (Brockes & Hall, 1975b). Extrajunctional receptors react preferentially, compared to junctional receptors, with anti-(AChR) antibodies from MG sera (Almon & Appel, 1975; Lindstrom et al., 1979a; Savage-Marengo et al., 1980; Dwyer et al., 1981a; Reiness & Hall, 1981), and with anti-(AChR) antibodies raised against purified receptor from denervated cat and chick muscle (Dolly et al., 1983). It has been suggested that the observed differences between the two receptor types may be due in part to carbohydrate residues (Dwyer et al., 1981b; Dwyer et al., 1981a; Hall et al., 1983; Turnbull et al., 1984). Studies on the phosphorylation of different AChR forms have also revealed differences between junctional and extrajunctional receptors (Saitoh & Changeux, 1981). There is no evidence that separate genes exist for the coding of the extra- and sub-synaptic forms of the receptor; observations concerning glycosylation and phosphorylation suggest that the differences may result from post-translational modifications to the receptor protein (Barnstable et al., 1983). Protein stability may be a consequence of its 3-dimensional structure (Goldberg & St. John, 1976), in which glycosyl residues have been proposed to have a stabilizing function (Olden et al., 1978). Enzymes effecting reversible phosphorylation

reactions in Torpedo electric organ have been demonstrated (Teichberg & Changeux, 1976; Saitoh & Changeux, 1980). It is therefore possible that such post-translational modifications may occur before the receptor is inserted in the membrane (Changeux & Danchin, 1976).

Synaptogenesis

During the development of the neuromuscular junction the AChR undergoes a series of complex changes, both in its distribution and in a number of its properties. In myotubes, AChRs initially occur over the entire muscle cell surface (Axelrod et al., 1976; Bevan & Steinbach, 1977). Functional contact of the myotube with nerves (Dennis et al., 1981) results in the appearance of clusters of receptors packed at high density at the sites of neuromuscular contact (Bevan & Steinbach, 1977; Braithwaite & Harris, 1979; Chow & Cohen, 1983); this coincides with a re-distribution of AChE activity (Bevan & Steinbach, 1977). During further development the number of AChRs at the end plate (junctional AChRs) increases, while the density of receptors in the extrajunctional membrane gradually decreases (Diamond & Miledi, 1962; Bevan & Steinbach, 1977), until in the adult, AChRs are found almost exclusively at the muscle end-plate (Axelsson & Thesleff, 1959; Miledi, 1960b; Hartzell & Famborough, 1972). For example, in the embryonic rat, extrajunctional AChRs are distributed with a density of $160/\mu m^2$ (at embryonic day 15); this decreases to $10/\mu m^2$ by embryonic day 19 and stays stable at this level after birth (Bevan & Steinbach, 1977). The maintenance of AChR clusters at the neuromusular junction is thought to depend upon specialized proteins which cause anchorage in the membrane (Bloch & Geiger, 1980; Hall, 1981; Prives et al.,

1982), the calcium ion concentration (Birnbaum et al., 1980; Bursztajn et al., 1984), the membrane fluidity (Bridgman & Nakajinia, 1981) and the rates of synthesis and degradation of the receptor (Bursztajn et al., 1983). The clustering of AChR molecules is thought not to effect the transition of the ion channel characteristic from long to short opening time (Berg & Hall, 1975; Burden, 1977b; Sakmann & Brenner, 1978).

The development of monoclonal and polyclonal AChR subunit-specific antibodies has recently allowed the clarification of discrete molecular events in the biosynthesis of AChR and its incorporation into the post-synaptic membrane (Anderson, 1983; Merlie et al., 1984). These studies have previously been limited because of the inability of the developing AChR to bind α-BGT. AChR subunits are translated independently from separate mRNAs, on membrane-bound ribosomes of the rough endoplasmic reticulum. This has been demonstrated by cell-free protein synthesis of Torpedo AChR subunits (Anderson & Blobel, 1981) in BC3H-1 mouse myoblast cell lines (Merlie et al., 1981) and confirmed by experiments using complementary DNA (cDNA) probes (Ballivet et al., 1982; Noda et al., 1982). The independant synthesis of the four subunits implies that they are, like other membrane and secretory proteins, synthesized with a signal peptide extension at their amino terminus (Blobel, 1980), and this has been shown in α,γ and δ subunits (Noda et al., 1982; Ballivet et al., 1982; Anderson et al., 1982). Asymmetrical insertion of these subunits into the membrane is a co-translational process directed by signal recognition particle (Walter & Blobel, 1982), which forms a high affinity 'bridge' between the nascent polypeptide chains and an integral membrane protein receptor in the rough endoplasmic reticulum (Gilmore et al., 1982; Meyer et al.,

1982), and decodes the information contained in the signal sequence. The complex then inititates translocation of the amino terminal portion of nascent polypeptide across the lipid bilayer. The signal protein is proteolytically removed following insertion into the membrane. During integration into the rough endoplasmic reticulum membrane, all four subunits receive asparagine-linked 'core' oligosaccharides side chains (Anderson & Blobel, 1981; Anderson et al., 1982; Merlie et al., 1981). The function of carbohydrate residues in the mature AChR is unknown, but it has been suggested that their presence may be related to the control of the rate of degradation of the molecules (Prives & Bar-Sagi, 1982). Assembly of the four subunits into a functional complex does not happen immediately after insertion into the rough endoplasmic reticulum membrane. Transport to the cell surface takes 2 - 3 hours, and is a temperature and energy dependant process (Devreotes et al., 1977), however, the exact site of heterologous AChR subunit assembly is unknown. Electron microscope autoradiography suggests that newly synthesized AChR is present in the Golgi apparatus where it resides for approximately 2 hours (Famborough & Devreotes, 1978; Bursztajn & Fischbach, 1984), demonstrating that integral membrane proteins follow the same intracellular transport pathway as secretory proteins (Palade, 1975). Post-translational events in AChR assembly have been studied by pulse-chase experiments using [^{35}S] methionine labelling of BC3H-1 cells in tissue culture, and have shown that conformational maturation occurs during the first thirty minutes after synthesis, where α subunits acquire the ability to bind α-BGT and be immunoprecipitated by monoclonal antibodies. Further post-translational modifications of glycosylation and/or phosphorylation of subunits may occur at this stage. Discrepancies

between the molecular weights of subunits obtained by SDS-PAGE of affinity purified AChR and those obtained as translation products of mRNA may suggest that the protein does undergo some form of processing (Marx, 1983).

Structural studies of membrane AChR by scanning transmission electron microscopy have indicated that each subunit borders the central channel (Wise et al., 1981), which implies that each subunit has a hydrophilic surface which must be maintained in the hydrocarbon interior of the membrane prior to oligomeric assembly. Evidence from experiments using cell-free systems suggests that AChR subunits may self-associate prior to complex formation, such associations would serve to stabilize hydrophilic surfaces of the subunits (Anderson & Blobel, 1983). No evidence for this in vitro has been documented. The results obtained by Mishina et al. (1984) show that assembly of all four subunits is required to elicit a normal nicotinic response to ACh; only the αsubunit is indispensable for α-BGT binding activity.

The change in immunological properties of rat muscle AChR from the embryonic to the adult form takes place during the first two post-natal weeks, concurrently with developmental changes in the mean channel open time (Reiness & Hall, 1981). At this stage the changes in AChR clustering and metabolic turnover time have already taken place.

The development of the human neuromuscular junction has not been so well documented. Structural studies of developing human muscle (foetal age 9 weeks to 9 months) have revealed that development is in two main stages:-

1. Up to 18 weeks, where two types of muscle cells - myoblasts and myotubes - are observed

2. After 18 weeks, where muscle fibres are similar to those observed in adult muscle (Minguetti & Mair, 1981).

The contribution of junctional and extrajunctional AChR types in this developmental scheme has not been documented.

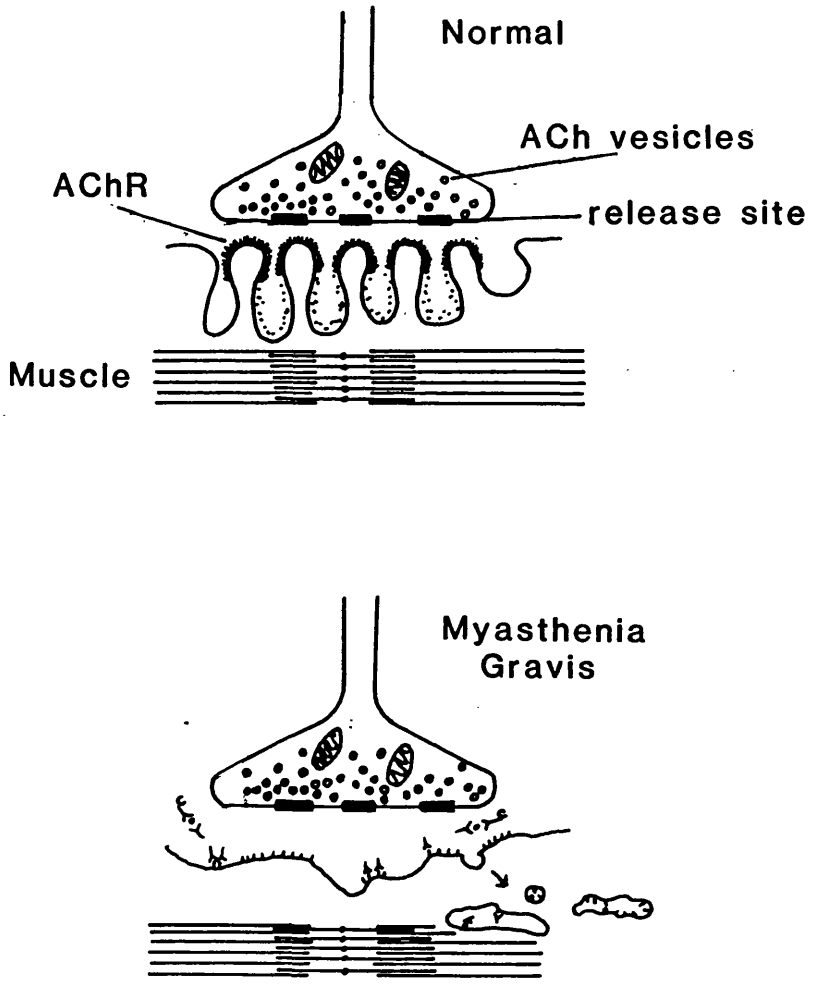
Brief history of myasthenia gravis

Jolly (1895) coined the name 'myasthenia gravis' for patients suffering from "grave muscle weakness" and showed that in these patients muscle strength was normal initially, but declined rapidly following exercise. By the end of the nineteenth century, about 70 cases of MG had been documented, most of them are described in summary form by Campbell & Bramwell (1900), who also proposed that the muscle weakness observed in MG patients might result from the presence of a circulating factor, possibly a toxin of microbial origin. The similarity of myasthenic symptoms to those of curare poisoning led Mary Walker (1934) to try the effects of phytostigmine, the anti-AChE antidote to curare poisoning. This provided the first evidence directly implicating an abnormality at the neuromuscular junction (see Figure 4) in MG patients; such a defect could be pre-synaptic, intra-synaptic or post-synaptic and various hypotheses were proposed as to the precise site and nature of the defect. Possible factors considered by various authors include the following:-

Pre-synaptic site:- failure in impulse propagation along the axon; impaired synthesis of ACh; faulty packaging of ACh in synaptic vesicles; impaired release of ACh; release of "false" neurotransmitter

Intra-synaptic site:- increased diffusion of ACh away from the synaptic cleft; prevention of ACh traversing the synaptic cleft

Figure 4. Diagram showing normal and myasthenic neuromuscular junctions



MG junction shows normal pre-synaptic terminal, but simplified post-synaptic membrane with reduced numbers of AChRs, sparse shallow synaptic folds where complement-mediated focal lysis results in shedding of membrane fragments (containing AChR and C3) into the synaptic cleft, and antigenic modulation, involving antibody cross-linking of AChR, internalization and proteolysis. The synaptic space is widened as a result of the loss of membrane.

Post-synaptic site:- combination of circulating blocking agent with ACh 'receptive substance'; depletion of ACh 'receptive substance'; increased AChE activity.

It was Simpson (1960) and Nastuk et al. (1959) who proposed that the ACh 'receptive substance' (then merely a hypothetical construct) was involved and postulated that it was blocked by an autoantibody. The elucidation of the precise site of the defect followed electrophysiological studies. Measurement of the response of whole muscle to repetitive supra-maximal stimulation of the nerve shows a significant decremental response in MG patients (Desmedt, 1966), which is consistent with the observed physical fatiguability of the myasthenic. Detailed studies revealed that the action potential generated by the nerve was normal, but that miniature end-plate potentials were reduced in MG patients (Elmquist et al., 1964; Santa et al., 1972; Albuquerque et al., 1976). Myasthenic motor end-plates also show reduced sensitivity to iontophoretically applied ACh in vitro (Rash et al., 1976). Furthermore, multi-electrode recordings from two or more muscle fibres from the same motor unit during a voluntary contraction showed a greater time delay between the two impulses in MG muscle fibres than in normal controls (Ekstedt & Stalberg, 1967). This results from the delay in onset of end-plate potentials which in turn results from the diminished size of miniature end-plate potentials. The location of the defect at the post-synaptic membrane was finally settled following the availibility of the post-synaptic α-neurotoxins (see "Introduction" p. 7) which bind with high specificity and affinity at the n-AChR in the post-synaptic membrane. Neuromuscular junctions of myasthenic patients show morphological alterations of flattening and simplification of post-synaptic folds (Engel & Santa, 1971) and

also bind far less iodinated α-BGT than normal controls (Famborough et al., 1973), indicating a reduced number of available AChR sites, which may be reduced to 30% of normal (see Figure 4). The area of the post-synaptic membrane that can bind α-BGT is also reduced (Engel et al., 1979), which reflects a decrease in the content of AChR rather than blocking of α-BGT binding to the receptor by some other factor (Lindstrom & Lambert, 1978). These physiological changes result in decreased efficiency of neuromuscular synaptic transmission, which, if reduced below threshold levels will result in failure to trigger action potentials. This will ultimately lead to reduction of muscle power and hence to the weakness and susceptibility to fatigue which are characteristic of the disease.

In contrast, the structure of the axon terminal has been shown to be retained, with the normal number of synaptic vesicles present and twice the normal concentration of ACh (Ito et al., 1976; Cull-Candy et al., 1978; 1980).

The autoimmune nature of MG was first suspected (Simpson, 1960; Nastuk et al., 1960) because of its association with other presumed autoimmune disorders (eg. Hashimoto's thyroiditis, systemic lupus erythrematosus, rheumatoid arthritis etc.), as well as because of structural abnormalities of the thymus found in approximately 75% myasthenic patients (Castleman, 1966). The serendipitous production of an experimental animal model for MG (Patrick & Lindstrom, 1973; Sugiyama, et al., 1973; Heilbronn & Mattson, 1974), obtained by immunization of rabbits with purified electric eel AChR, substantiated this hypothesis, providing evidence that an autoimmune response directed against AChR can produce many of the features characteristic of MG. This suggested that antibody directed against AChR might be found in MG patients, but early attempts to

demonstrate such a 'circulating factor', which interfered with neuromuscular transmission, were unsuccessful and gave rise to conflicting results (Wilson & Stoner, 1944; Lammers & Van Spijk, 1954; Nastuk, et al., 1959; McFarlin et al., 1966; Parkes & McKinna, 1967; Namba & Grob, 1969). It was not until 1974 that Almon et al. were able to show that approximately 50% of MG sera tested could inhibit α-BGT binding to rat denervated AChR preparations. The factor responsible for this observation was later identified as Immunoglobulin G (IgG) (Almon & Appel, 1975). Shortly after this, Bender et al. (1975) showed that peroxidase-labelled myasthenic IgG inhibited the binding of α-BGT to normal human muscle, and Aharonov et al. (1975) showed that in 80% of MG patients, complement fixation could be effected in the presence of small quantities of Torpedo AChR.

The question as to whether circulating antibodies are pathogenic, or merely represent a secondary response to AChR damage caused by some other agent, is critical in understanding the pathogenesis of MG, and a number of observations are relevant to this. Toyka et al. (1977) showed that passive transfer of myasthenic IgG into mice induces characteristic features of MG. Plasma exchange of MG patients causes temporary improvements in their muscle strength, which parallels the decrease in the concentration of serum antibodies (Pinching et al., 1976; Dau et al., 1977). Placental transfer of anti-(AChR) antibodies from a myasthenic mother to the foetus causes transient neonatal MG, with an antibody level at birth similar to that of the mother (Keesey et al., 1977). The antibody level declines with a $T_{\frac{1}{2}}$ of 8 days; full recovery is noticed after 3 weeks. These observations clearly demonstrate the involvement of a circulating factor as a primary agent in the disease process.

Mechanisms for the pathogenesis of anti-(AChR) antibodies

In order to cause synaptic dysfunction, antibodies need to leave the vascular system, diffuse into the extra-cellular space, enter the synaptic cleft and reach AChR on the post-synaptic folds. Zurn & Fulpius (1976) used complexes of α-BGT covalently coupled to IgG to show that molecules the size of antibodies can reach the AChR in situ in mouse diaphragm. Localization of IgG at end-plates has also been reported by Engel et al. (1977a) using peroxidase-labelled Staphylococcal protein A (which binds to the Fc portion of human IgG subclasses 1,2 and 4) and by Sahashi et al. (1980). Although these results alone do not necessarily implicate antibodies in a primary pathogenic role in MG, there is evidence that circulating anti-(AChR) antibodies may be pathogenic in one or more of three different ways:-

(i) by altering the rate of turnover of AChR

(ii) by blocking the active site of AChR

(iii) by complement-mediated membrane destruction

These will be discussed in turn.

Increased degradation of AChR

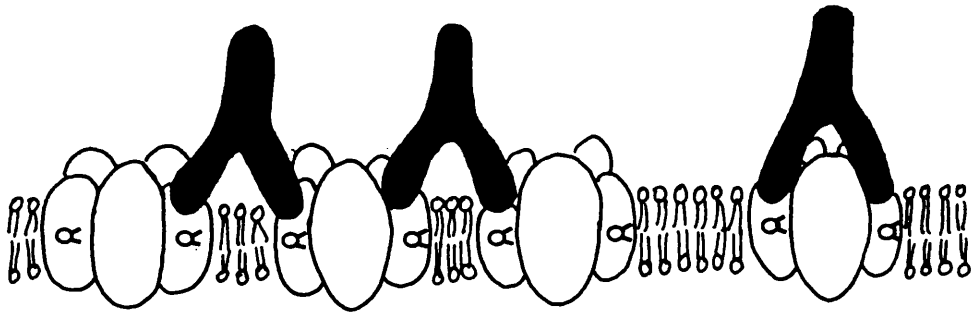
IgG from myasthenic sera has been shown to accelerate the degradation of AChRs in rat (Anwyl et al., 1977; Kao & Drachman, 1977b) and human (Bevan et al, 1977; Drachman et al., 1981) muscle tissue culture systems; the rate increasing 2 - 3 fold above that of normal controls (Kao & Drachman, 1977b). Significant acceleration of AChR degradation in this system was observed in 66% (Conti-Tronconi et al., 1981) and 90% (Drachman et al., 1982) of myasthenic patients studied. Conti-Tronconi et al. (1981) found, in addition, that

increased degradation was proportional to the severity of the disease state (see also Drachman et al., 1981), and was related to the antibody titre (if the titre was less than 10 nM) (see also Appel & Elias, 1979). Increased degradation is dependent on the ability of IgG to cross link receptors (Drachman et al., 1978; Conti-Tronconi et al., 1981), which is possible because of their divalent nature (see Figure 5). Monovalent Fab fragments are inactive, except when they are themselves cross-bridged by a second antibody (Prives et al., 1979; Drachman et al., 1978; Lindstrom & Einarson, 1979). Morphological studies indicate that such cross-linking causes re-distribution of AChR in the membrane, followed by enhanced endocytosis. Aggregation of AChRs in muscle cultures following the addition of myasthenic serum has also been observed by fluorescence microscopy, autoradiography (Lennon, 1978; Tarrab-Hazdai et al., 1979) and by freeze-fracture electron microscope studies (Pumplin & Drachman, 1983). Antibody-induced acceleration of AChR degradation has been described in sections of normal adult skeletal muscle (Merlie et al., 1979; Reiness et al., 1978; Stanley & Drachman, 1978) indicating that this is not purely a feature of extra-junctional receptors. More recent results from Wilson et al. (1983a,b) have shown that synthesis of diaphragm end-plate AChRs is increased in vivo, suggesting the existance of a compensatory mechanism serving to stabilize the number of AChRs.

Blockade of AChR

Anti-(AChR) antibodies from MG sera may inhibit neuromuscular transmission by pharmacological blockade of AChRs; however, the extent of such a contribution to the pathogenesis of MG is still a controversial matter. Electrophysiological studies have shown that

Figure 5. Diagram showing inter- and intra-molecular cross-linking of AChR by anti-(AChR) antibodies



Inter-molecular cross-linking

Intra-molecular cross-linking

immunoglobulins from sera with MG (Anwyl et al., 1977; Bevan et al., 1977) and animals with EAMG (Patrick et al., 1973) cause reduced sensitivity of muscle cells in culture to applied ACh. Such immunoglobulins have also long been known to block binding of [^{125}I] α-BGT to detergent solubilized AChR; an effect that has served as a method of identifying antibodies directed at (or near) the cholinergic binding site (Almon et al., 1974a; Shibuya et al., 1978; Ito et al., 1978; Vincent & Newsom-Davis, 1979; Mittag et al., 1981). Reports concerning the incidence of antibodies with this specificity vary greatly - the proportion of MG patients who have anti-(ACh binding site) antibodies has been reported as ranging from 7 - 90% (Almon et al., 1974a; Vincent & Newsom-Davis, 1979; Drachman et al., 1980; Drachman et al., 1982); while the proportion of such antibodies in an individual patient has been quoted as varying from 0 - 100% of the total anti-(AChR) antibodies (Whiting et al., 1983; Dwyer et al., 1979). Attempts to relate anti-(ACh binding site) antibodies to the clinical severity of MG symtoms have proved unsuccessful so far (Lindstrom et al., 1976a; Lefvert et al., 1978; Vincent & Newsom-Davis, 1979), although these antibodies have been implicated in the pathogenesis of EAMG (Zurn & Fulpius, 1977; Gomez & Richman, 1983).

Care must be taken in drawing conclusions from these data, as the ability of a particular serum to block ligand binding to the receptor in situ and to the solubilized receptor may differ (Sanders et al., 1976; Karlin et al., 1978). Furthermore, blockade of [^{125}I]α-BGT binding does not necessarily parallel the blockade of ACh binding in vivo, as the small molecular weight of ACh (182 compared to 8000 for α-BGT) may allow greater accessibility to the ACh binding site, even in the presence of antibody.

Complement-mediated destruction of muscle membrane

The simplified post-synaptic structure observed in myasthenic patients has been attributed to the complement-mediated destructive effects of anti-(AChR) antibodies (Engel et al., 1979; Toyka et al., 1977). Published evidence for muscle cell lysis is largely indirect, relying mainly on the demonstration of IgG and C3 binding to the post-synaptic membrane (Engel et al., 1977a; Sahashi et al., 1978; 1980). The final lytic component, C9, is found both on the post-synaptic membrane and also on degenerated membrane in the synaptic space (Engel et al., 1979; Engel & Fumigalli, 1982). Direct measurement of antibody-mediated lysis of muscle cells has been more difficult to demonstrate because of the lack, until recently, of a sufficiently sensitive and specific method for quantitating such lysis. Cambridge & Stern (1981) reported a procedure for measurement of myotube-specific toxicity, dependent on the selective uptake of [^{3}H]-carnitine which is subsequently released following cell lysis. Using this method, Childs et al. (1984) have shown that eight out of thirteen myasthenic sera (and zero out of nine controls) showed myolytic activity in monolayer rat muscle cultures, indicating that complement-mediated mechanisms could be responsible, at least in some myasthenic patients, for the known post-synaptic membrane damage. Alterations of membranes by such means would be expected to reduce the efficiency of transmission and could contribute to the other factors which reduce neuromuscular transmission in MG.

Present evidence suggests that each of the immune mechanisms mentioned above is involved to some extent in individual patients, but their relative roles in the pathogenesis of the disease is still

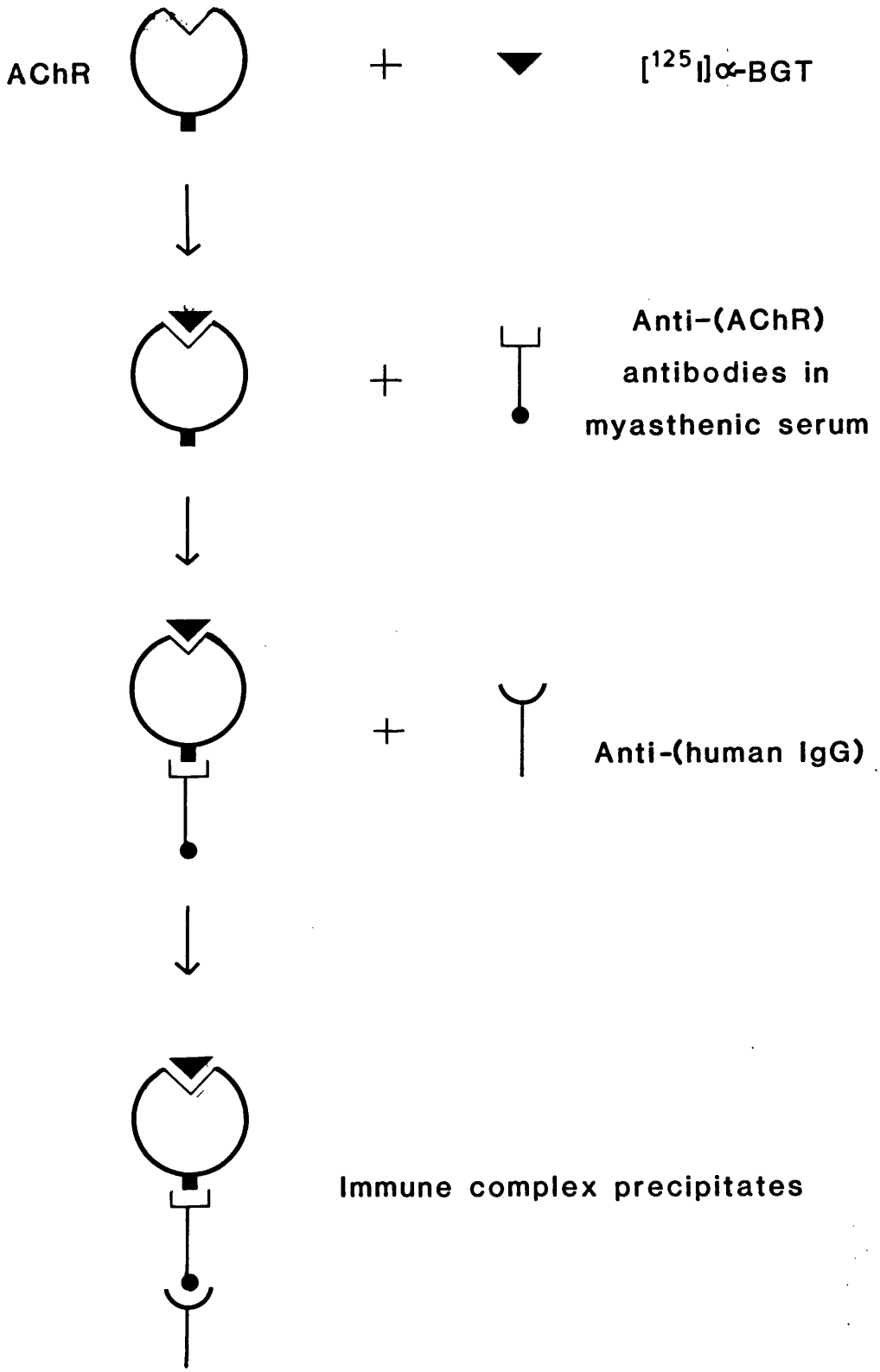
a controversial matter.

Circulating anti-(AChR) antibodies could also be pathogenic as immune complexes; their deposition would lead to widespread generalized damage. Methods for their detection are, however, largely non-specific, and further information concerning their role, if any, in MG must await the development of specific assay methods.

Quantitation of anti-(AChR) antibodies

Following the discovery of the presence of anti-(AChR) antibodies in the sera of myasthenic patients (Almon et al., 1974a), a radioimmunoassay for their quantitation soon became a standard diagnostic procedure (Lindstrom et al., 1976; Lindstrom, 1977; Monnier & Fulpius, 1977; Newsom-Davis et al., 1978; Dwyer et al., 1979; Tindall et al., 1981; Carter et al., 1981). This method is based on a direct immunoassay developed by Patrick et al. (1973) for the quantitation of anti-(AChR) antibodies induced by injection of electric eel AChR into rabbits. It uses, as antigen, crude detergent extracts of amputated human limb muscle in which the AChR is labelled with [^{125}I]α-BGT. The labelled extract is incubated with MG serum, followed by goat anti-(human IgG) antiserum and further incubation. The resulting precipitate is pelleted, washed, counted and the amount of specific antibody binding expressed as nmoles/l specific α-BGT binding sites (see Figure 6). 70 - 95% of clinically diagnosed MG patient have detectable anti-(AChR) antibodies (Appel et al., 1975; Lindstrom et al., 1976a; Mittag et al., 1981; 1984; Monnier & Fulpius, 1977; Ito et al., 1978; Lefvert et al., 1978; Bradley et al., 1978), showing values ranging from 0 - 844 nM. Control values, including normals, patients with other neurological disorders (eg. diabetic neuropathy, Friedreich's ataxia, myotonic

Figure 6. Diagrammatic representation of the radioimmunoassay for anti-(AChR) antibodies



dystrophies, Lambert-Eaton myasthenic syndrome, Guillain-Barre syndrome etc.) and patients with other autoimmune diseases (eg. polymyositosis, Sjogren's syndrome, systemic lupus erythrematosus etc.), were in the range 0 - 6 nM.

Anti-(AChR) antibodies have been detected in MG associated with D-penicillamine treatment for rheumatoid arthritis or Wilson's disease (Masters et al., 1977; Russell & Lindstrom, 1978). Most cases improve rapidly after cessation of penicillamine treatment, and this is accompanied by a decrease in the antibody level (Vincent et al., 1978a). Penicillamine may disturb the maintenance of tolerance in some way; attempts to test this in experimental animals have so far proved unsuccessful (Russell & Lindstrom, 1978).

The anti-(AChR) antibody assay described above is specific for IgG, but other immunoglobulins can be detected with different anti-Ig precipitating sera (Tindall, 1981), although only concurrently with IgG and in low concentrations. There is no direct evidence for different pathogenic roles for the different Ig types, despite the fact that IgM is known to appear in the early phase of an immunization procedure. Lefvert et al. (1978) observed that in three patients with short duration of myasthenic symptoms, IgM antibodies were present initially, but not IgG antibodies. This situation was, however, reversed during the course of the disease, when IgM antibodies disappeared as IgG antibodies appeared. This finding was not confirmed by Tindall (1981).

Heterogeneity of anti-(AChR) antibodies

The AChR exists in detergent solution as a large macromolecule with several antigenic domains. The variable sizes of AChR - anti-(AChR) antibody complexes observed by gel filtration (Vincent & Newsom-Davis, 1979; Dwyer et al., 1979) and the broad peaks of anti-(AChR) activity obtained on isoelectric focussing of myasthenic IgG (Lefvert & Bergstrom, 1978) suggest that different sera contain antibodies of differing specificities. Such experiments indicate that anti-(AChR) antibody is polyclonal in most patients. Additional evidence for this comes from the use of IgG subclass specific antisera in the assay for anti-(AChR) antibodies, where Vincent & Newsom-Davis (1980) show that most patients have anti-(AChR) antibodies in subclasses 1,2 and 4, and that occasionally IgG3 predominates. This is in agreement with the observations of Tindall (1981) who found the same anti-(AChR) antibody titre using either Staphylococcal protein A or anti-(human IgG) antiserum to precipitate AChR-antibody complexes; but contrasts with the findings of Lefvert & Bergstrom (1978) who suggested that IgG3 is the dominant form of anti-(AChR) antibody.

The diversity of anti-(AChR) antibodies is also suggested by the variation seen between individual sera in their cross-reactivity with AChR preparations from different species (Lindstrom et al., 1978a; McAdams & Roses, 1980; Savage-Marengo et al., 1979; 1980; Harrison et al., 1981; Zielinski et al., 1982) and also from different muscles of the same species (Vincent & Newsom-Davis, 1979; Compston et al., 1980; Almon & Appel, 1975; Weinberg & Hall, 1979). The abilities of MG sera to inhibit binding of [^{125}I]α-BGT to

detergent solubilized AChR also show similar variable characteristics (see "Introduction" p. 25). Anti-(ACh binding site) antibodies are found in 7 - 90% patients, and the proportion of antibodies inhibiting α-BGT binding as a percentage of those which do not interfere with the α-BGT binding site varies from 0 - 100% (Whiting et al., 1983; Dwyer et al., 1979).

The nature of the antigenic domains on AChR responsible for variable reactivity with anti-(AChR) antibodies is not known. However, the recent advances in monoclonal antibody technology have enabled the production of monoclonal antibodies (Kohler & Millstein, 1975) to purified AChR. These are being used to probe the structure and function of AChR, and, indirectly, to define the binding sites of anti-(AChR) antibodies to the receptor (Tzartos & Lindstrom, 1980; Vincent, 1983). The majority of antibodies (60 - 70%) bind to a main immunogenic region on the α subunit, although a proportion of antibodies may be directed towards the β and δ subunits which lie next to the α subunit (Tzartos & Lindstrom, 1980; Tzartos et al., 1981; 1982; 1983).

Relationship between anti-(AChR) antibody titre and disease severity

The discovery of circulating anti-(AChR) antibodies was exciting from a clinician's point of view, as a positive correlation between the level of antibody and the clinical status of the MG patient would permit assessment of the progression of the disease, predict the occurrence of relapses and enable monitoring of the effectiveness and benefits of a particular treatment regime. Serial assays performed on serum samples from a given MG patient show titres which reflect the clinical state of the patient; this has been most convincingly demonstrated in patients treated by plasma

exchange combined with immunosuppressive therapy (Newsom-Davis et al., 1978; 1979; Carter et al., 1980). Continued post-exchange alleviation of MG symptoms was paralleled by lowered anti-(AChR) antibody titres and, if relapse did occur, was associated with a sharp rise in antibody titre (Carter et al., 1980).

Between patients, however, the level of antibody does not correlate well with the clinical status of the patient (Ito et al., 1978; Lefvert et al., 1978; Lindstrom et al., 1976a). A high percentage of positive anti-(AChR) antibody titres are found in patients with generalized MG, but many patients in clinical remission also have antibody titres well within the range of those with active disease (Vincent & Newsom-Davis, 1979). A further number of myasthenic patients have no detectable antibody (Lindstrom, 1977), but have severe generalized MG. Many patients with low titres have predominantly ocular symptoms (Ito et al., 1978; Lindstrom et al., 1976a; Compston et al., 1980) and a proportion of these are within the statistical limits of control values. The ability to detect low titres reliably, and hence discriminate between MG and normal sera, depends on the lower cutoff value used. The variation seen in the percentage of MG patients reported to have significant anti-(AChR) antibody titres may well be a reflection of the different lower cutoff values used by different workers (Mittag et al., 1984).

The lack of correlation between antibody titre and disease severity can be explained in a number of ways, including the following:-

- the immunoprecipitiation assay used does not detect all antibodies present (eg. anti-(ACh binding site) antibodies)
- the antibodies detected may not necessarily be pathogenic in

the disease

- the levels of circulating antibodies may not reflect levels at the neuromuscular junction
- antibodies circulating as immune complexes escape detection
- the half-life of IgG varies between subclasses

Comparisons of results obtained are further complicated by the different sources of antigen used in the assay. Modifications to the assay include the use of denervated rat muscle AChR as antigen (Mittag et al., 1978; 1981; 1984), the advantage of denervation being that after 8 - 10 days a 10 - 50 fold increase in the amount of AChR is found resulting from the development of extrajunctional AChRs over the surface of the muscle membrane (Dolly, 1979; see also "Introduction" p. 10). Although sera from many MG patients react with rat AChR, the antibody titres obtained are lower and the percentage of negative results increases when this antigen is used (Savage-Marengo et al., 1979). It is now generally accepted that human muscle AChR is the preferred antigen for use in the RIA, although this presents a problem in obtaining a regular and consistent source, and there is some controversy in the literature concerning the nature of human muscle used for such studies. Further thought is given to this problem in the "Discussion" section. Although the use of rat antigens is not recommended for use in the RIA, the results obtained from such experiments may be useful for giving information about antigenic sites which may be conserved and therefore represent functionally important domains on the AChR.

Thymic involvement in MG

The high incidence of thymoma, thymic hyperplasia (Castleman, 1966) and germinal centres in MG has drawn attention to the role of the thymus in MG, and it has been suggested that the thymus may be the source of the autoimmune response (Lindstrom, 1979). It is now well established that the thymus is responsible for the development of immunocompetent T cells, which are involved in both regulatory and effector functions. Non-specific and antigen specific T cell mediated help and suppression of Ig synthesis in human peripheral blood lymphocytes has been reported; and it considered that this regulation is important in normal immune mechanisms. Breakdown of regulation may play a role in the aetiology of immune disorders, especially those of an autoimmune nature. The production of autoantibodies in MG suggests that the normal immunoregulatory mechanisms may be altered in some way. Thymectomy is one of the effective therapies in the management of myasthenia (although its value is continually debated (McQuillen & Leone, 1977; Vincent & Newsom-Davis, 1982a)), and may (Lefvert et al., 1978; Scadding et al., 1977) or may not (Roses et al., 1981; Olanow & Roses, 1981a; Olanow et al., 1982) be accompanied by a decrease in the level of circulating serum anti-(AChR) antibodies. Although it has been suggested (Olanow & Roses, 1981b) that a 'thymic factor' is critical to the development of clinical weakness in MG, the role of the thymus in the pathogenesis of the disease is not understood. The thymus is known to be a site of AChR antigen in rats (Lindstrom et al., 1976b) and humans (Henry, 1972); thymic epithelial cells bear AChR (Engel et al., 1977b); so too do thymic lymphocytes (Fuchs et al., 1980). Furthermore myoid cells in tissue culture from rat and human thymus also express AChR (Kao & Drachman, 1977a). There is

evidence that the thymus is a site of antibody production (Mittag et al., 1976). Germinal centres contain cells exhibiting B cell markers (Abdou et al., 1974; Staber et al., 1975); thymic cells from MG patients can spontaneously synthesis anti-(AChR) antibody in culture (Vincent et al., 1978b; Newsom-Davis et al., 198la,b) and can enhance anti-(AChR) antibody production by autologous peripheral blood lymphocytes (Newsom-Davis et al., 198la,b; Willcox et al., 1984). However, the rates of anti-(AChR) antibody synthesis in culture do not suggest that the thymus is a major site of antibody production.

Cell-mediated immunity

While it is well established that the humoral immune system, via anti-(AChR) antibodies, plays a central role in the pathogenesis of MG (Vincent, 1980), the relative role of the cellular immune response is much less clearly defined. Peripheral blood lymphocytes from MG patients can be stimulated to undergo blast transformation in the presence of muscle AChR from electric eel (Abramsky et al., 1975; Richman et al., 1976), electric ray (Conti-Tronconi et al., 1977; Hohlfeld et al., 1984) and foetal calf (Sinigaglia et al., 1984); but the corresponding experiments using human AChR have not been convincing (McQuillen et al., 1983), possibly because of the difficulty in obtaining sufficient quantity of AChR. Stimulation of lymphocytes in response to a specific antigen indicates that the cells have been previously sensitized to that antigen. Lymphocytic sensitization to AChR *in vitro* could indicate two different functions that might be carried out in patients by specifically primed T cells:- (i) regulation of synthesis of anti-(AChR) antibodies (ii) direct action of specific cytotoxic lymphocytes in

the destruction of the neuromuscular junction. Altered regulation of immunosuppressive function is associated with altered suppressor cell activity in various diseases and especially in autoimmune and immunodeficient states (Waldeman et al., 1976; Abdou et al., 1976), and a decrease in suppressor activity has been suggested in MG (Kelley et al., 1981; Mishak & Dau, 1981). However, as with humoral immunity, there has been found no correlation between cell-mediated effects and the clinical status of MG patients.

Origin of the autoimmune response in MG

While the pathogenesis of MG is well documented and becoming increasingly understood, the reasons for its initiation are still speculative. In MG, as in other autoimune disorders, the immune system responds to self components in an inappropriate way. This may result from :-

1. An alteration in the antigen causing it to be perceived as 'non-self' eg. shedding of receptor from the muscle surface; viral infection causing modification of surface protein; exposure of normally sequestered antigens
2. Defective control of the immune system, allowing it to respond to normal antigen eg. failure to eliminate antigen-sensitive cells during development, monoclonal proliferation of antigen-sensitive cells or non-specific stimulation of the immune system by viral or microbial infection leading to imbalance of control mechanisms.
3. A combination of 1 and 2.
4. The presence of anti-iodiotype antibodies against anti-viral antibodies which have the incidental properties of being antibodies to host structures.

Compston et al. (1980) have distinguished three distinct groups of MG patients, based on thymic pathology and age of onset:-

Group A - thymoma

Group B - non-thymoma; age of onset less than 40 years

Group C - non-thymoma; age of onset greater than 40 years

Between these groups there were highly significant differences in mean anti-(AChR) antibody levels - the highest level being in Group A and the lowest in Group C. HLA-A1, B8 and/or DRw3 histocompatibility antigens were strongly associated with Group B; A3, B7 and/or DRw2 with Group C, but none in particular with Group A. Other differences between the three groups were visible in the sex incidence; in the proportions with anti-(striated muscle) antibody and other autoantibodies; in the incidence of other autoimmune disorders and in their response to thymectomy and immunosuppressive drugs. One further sub-group may comprise those patients with restricted ocular disease - a large proportion of this group do not have detectable anti-(AChR) antibody and the ratio of anti-(ocular AChR) antibody to anti-(limb AChR) antibody is higher than in those with generalized MG (Vincent & Newsom-Davis, 1979). This disease heterogeneity implies diverse origins for the disease, which may result from a genetic pre-disposition to an altered autoimmune response.

AIMS OF THE PROJECT

In view of the limited correlation observed between anti-(AChR) antibody titre and disease severity in MG patients (Lindstrom et al., 1976), and the results of preliminary investigations in the laboratory which had revealed a patient-specific antibody pattern in a group of MG patients, the nature of the heterogeneity of anti-(AChR) antibodies in the sera of MG patients was investigated with the aim of elucidating possible correlations between levels of specific antibody sub-populations in the disease, both in individual patients and between patients. If such correlations are present they may help clarify the role of humoral antibodies in MG.

The role of anti-(ACh binding site) antibodies in the disease remains unclear (Vincent, 1980), the antibody-induced release of bound [^{125}I]α-BGT from [^{125}I]α-BGT labelled AChR complexes was therefore examined. This may help to identify a pathologically relevant antibody sub-population in MG sera.

Finally, studies were made to compare the interaction of α-BGT and anti-(AChR) antibodies with foetal and adult AChR, as differential reactions between the two receptor types may help identify the nature of the autoimmunogen in MG.

MATERIALS

Radiochemicals

Carrier-free Na[^{125}I] in dilute NaOH (100 mCi/ml) was from Amersham International (Amersham, Bucks, U.K.) and was stored at ambient temperature for not longer than three weeks after its activity reference date.

[^{125}I]α-BGT in 0.05 M phosphate buffer containing BSA (5 mg/ml) at specific activity greater than 200 Ci/mmol was from Amersham International (Amersham, Bucks, U.K.), or was prepared in our laboratory (see "Methods" section 1.1), and was stored at ambient temperature for not longer than three weeks after its activity reference date.

N-[propionyl-^{3}H]-propionylated-α-BGT ([^{3}H]α-BGT) in aqueous solution containing 0.02% (w/v) BSA at 40 - 70 Ci/mmol was from Amersham International (Amersham, Bucks, U.K.) and was stored at -20^{o}C.

Ligands

α-BGT from *Bungarus multicinctus* was purchased from Boehringer Corps., Mannheim, W. Germany, or from Sigma Chemical Co., Kingston-upon-Thames, U.K., as a lyophilised powder.

Naja naja siamensis venom was obtained from the Miami Serpentarium, Florida, U.S.A.

Benzoquinonium chloride was a generous gift from Stirling Winthrop Inc., Rensselaer, N.Y., U.S.A.

FITC-α-BGT was from Sigma Chemical Co., Kingston-upon Thames, U.K.

Sources of AChR

Human adult muscle was supplied by the Royal United Hospital, Bath; St. Martins Hospital, Bath and the Bristol Royal Infirmary, and was obtained from lower limb amputations resulting from severe vascular disorders or from road traffic accidents. Within fifteen minutes of amputation, calf muscle was crudely dissected free from fat, tendon and skin, transported in ice, solid carbon dioxide or liquid nitrogen and stored at -80°C for up to six months.

Human foetuses (foetal age 9 - 22 weeks) were supplied by the Royal United Hospital, Bath; the Bristol Royal Infirmary; the General Hospital, Bristol and the Bristol Public Health Laboratory, and were obtained by suction on a vacuum line or from prostaglandin-induced terminations. Foetuses were frozen as soon as possible. Limb, back and intercostal muscles were dissected prior to use.

Torpedo marmorata electroplax were purchased from Institut de Biologie Marine, Arcachon, France and stored at -80°C.

Three-month old female Wistar rats were used as a source of muscle for both junctional and extra-junctional receptor preparations (see "Methods" section 4.2).

Immunoreagents

Sera from normal volunteers were obtained from colleagues in the department. Myasthenic serum samples were obtained from several hospitals in the U.K. as samples received for the routine assay of anti-(AChR) antibodies. Sera were frozen and stored at -20°C.

Goat anti-(human IgG) antiserum was prepared by repeated intramuscular injections of purified human IgG into goat, as

described in the "Methods" section (6).

Alkaline phosphatase conjugated to anti-(human IgG) antibodies (rabbit, lyophilised) was obtained from Sigma Chemical Co., Kingston-upon-Thames, U.K.

Rabbit anti-(α-BGT) antiserum was a generous gift from Jean Whyte.

Normal sheep serum was a generous gift from Ahmed Jehanli, to whom I am also grateful for helpful discussion concerning the methodology of the immunohistochemical studies.

Sheep anti-(rabbit IgG) antibodies, and Horseradish Peroxidase anti-Peroxidase (PAP) complex (rabbit) were from Miles Labs., Stoke Poges, Slough, England.

Freunds complete and incomplete adjuvants were from Miles Labs., Stoke Poges, Slough, England.

Chemicals

Standard Laboratory Reagents were from Sigma Chemical Co., Kingston-upon-Thames, U.K. or B.D.H. Chemicals, Poole, Dorset, U.K.

Gel filtration reagents were supplied by Pharmacia Ltd., Hounslow, U.K.

Ion-exchange resins, DEAE-cellulose filter discs and GFC glass fibre filter discs were from Whatman Lab. Sales Ltd., Maidstone, Kent, U.K.

Soluene-350 tissue solubilizer was from Packard, Reading, Berks.

Tissue Tek embedding medium for frozen tissue sections was obtained from Lab. Tech. Products, Division Miles Inc., Naperville, Illinois, U.S.A.

Counting instruments

[^{125}I] was counted in an LKB 1280 Ultrogamma counter. Tritium was counted in a tritium-specific channel in a Packard Tri-Carb Scintillation counter (model 3255) for a two-minute counting period. Correction for quenching was made using the channels ratio method.

METHODS

1. Iodination and characterization of α-BGT.

1.1 Preparation of [^{125}I]-labelled α-BGT

α-BGT was labelled with [^{125}I] by the chloramine-T method (Hunter, 1967) as modified by Urbaniak et al. (1973). Carrier-free Na[^{125}I](100 mCi/ml) in dilute sodium hydroxide (10 µl) was added to α-BGT (10 µg) in 0.05 M potassium phosphate buffer, pH7.5 (20 µl), followed by 0.5% (w/v) chloramine-T in 0.05 M potassium phosphate buffer, pH7.5 (10 µl). The mixture was stirred at room temperature for 1 min at 23°C, after which the reaction was terminated by the addition of 0.016% (w/v) sodium metabisulphite in 0.05 M potassium phosphate buffer, pH7.5 (750 µl), followed by 1% (w/v) carrier potassium iodide in 0.05 M potassium phosphate buffer, pH7.5 (200 µl). The labelled protein was separated from unreacted [^{125}I] by passage through a column of Sephadex G-25 (25 x 1 cm) previously equilibrated in 10 mM potassium phosphate buffer, pH7.5, containing 1% (w/v) BSA. Fractions (1 ml) were collected and aliquots (5 µl) from these fractions were counted for radioactivity. The peak fractions containing [^{125}I]α-BGT were pooled and stored at 4°C for not longer than 3 weeks. The specific radioactivity of the [^{125}I] α-BGT was calculated assuming 100% recovery of protein. The biological activity of the toxin and the amount of iodinated protein precipitated by trichloroacetic acid were also determined, as described below.

1.2 Fractionation of [^{125}I]-labelled α-BGT species

The mono- and di-iodinated α-BGT species were separated by ion-exchange chromatography by using a method adapted from those of Vogel et al. (1972) and James et al. (1980). α-BGT was iodinated in the usual way (see "Methods" section 1.1), except that chloramine-T, sodium metabisulphite and potassium iodide were made up in 3.3 mM potassium phosphate buffer, pH7.5. The iodination products were diluted 2-fold in elution buffer (3.3 mM potassium phosphate buffer, pH7.5 containing 0.1% (w/v) BSA and 0.01% (w/v) sodium azide), and applied to a column (1 ml) of CM-Sephadex C-50 which had been pre-equilibrated in elution buffer. The column was washed extensively with elution buffer alone, then with elution buffer containing 10 mM sodium chloride and finally with elution buffer containing 100 mM sodium chloride. The flow rate throughout was 15 ml/h and fractions of 1 ml were collected. Samples (5 µl) from each fraction were taken and counted for radioactivity. The iodinated toxins of the 2 peaks were pooled separately and stored at 4°C.

1.3 Determination of the concentration of iodinated α-BGT species by dilution assay

The concentrations of iodinated α-BGT species obtained from fractionation of [^{125}I]α-BGT on CM-Sephadex C-50 (see "Methods" section 1.2) were determined by a dilution assay similar to that described by James et al. (1980). A constant volume (5 µl) of each iodinated α-BGT species was diluted with various known concentrations of α-BGT (0, 4, 8, 12 pmol per test sample, diluted from 0.125 µM stock), and the binding to solubilized Torpedo AChR (0.1, 0.2, 0.3, 0.4 pmol per test sample, diluted from 25 nM stock) was measured. The total α-BGT concentration was always at least

10-fold in excess of the receptor concentration, and the total volume of each sample was 150 µl, all dilutions being made in toxin binding assay buffer (see "Methods" section 7.2). After incubation for 90 min at 23°C (or overnight at 4°C), each sample was applied to 2 DEAE-81 cellulose filter discs (diamter 24 mm; pre-moistened with toxin binding assay buffer), left to stand for 2 min, followed by vacuum filtration in a Millipore filter unit. The filters were washed (3 x 1 ml) with toxin binding assay buffer and counted for radioactivity. Non-specific binding was measured as described for the toxin binding assay (see "Methods" section 7.2).

1.4 Determination of the biological activity of radiolabelled α-BGT species.

The biological activities of radiolabelled α-BGT species were determined by 2 different methods, each involving the measurement of the proportion of radiolabelled α-BGT that can be bound by a large molar excess of purified AChR from Torpedo marmorata. Duplicate samples of Torpedo AChR (20 pmol, 100 µl) were incubated with the test radiolabelled α-BGT species (0.2 pmol, 50 µl) for 90 min at 23°C in the presence and absence of 1mM benzoquinonium chloride. The first assay method involves separation of bound and free species by ion-exchange chromatography in a DEAE cellulose filtration assay, as described in the "Methods" section 7.2. The second method for separation of bound and free species was by gel filtration on Sephadex G-50. Torpedo AChR was incubated with the radiolabelled α-BGT species as described above, after which each sample was then applied to a column (25 x 1 cm) of Sephadex G-50 pre-equilibrated in toxin binding assay buffer (see "Methods" section 7.2. Fractions (1 ml) were collected and counted for radioactivity. When using [^{3}H]

α-BGT, the methods of assay were as described above, except that DEAE-81 filter discs were dried, then added to scintillant (10ml) which contained 10% (v/v) Soluene-350, 0.45% (w/v) PPO, 27% (v/v) Triton X-100 and 63% (v/v) toluene. Aqueous samples were counted in 0.5% (w/v) PPO, 30% (v/v) Triton X-100 and 70% (v/v) toluene. The biological activities of the radiolabelled α-BGT species were expressed as a percentage of the total counts added.

1.5 Determination of the radiolabelled protein by precipitation with trichloroacetic acid.

The radiolabelled α-BGT species were diluted 1 in 100 with 10 mM potassium phosphate buffer, pH7.4, containing 1% (w/v) BSA. Triplicate samples of the diluted toxin (100 µl) were placed on ice and 12% (w/v) cold trichloroacetic acid (100 µl) added. After 30 min, the precipitates were collected by vacuum filtration on a Whatman GFC glass fibre filter discs, washed with 6% (w/v) cold trichloroacetic acid (5 ml) and counted for radioactivity. Discs containing [^{3}H]α-BGT were treated as described in the previous section. Acid precipitable counts were expressed as a percentage of the total counts added.

2. Purification of the α-toxin from venom of Naja naja siamensis.

α-Toxin was purified from the crude venom of Naja naja siamensis by modification of the method of Cooper and Reich (1972). Crude venom (1 g) was dissolved in distilled water (10 ml) and loaded onto a phosphocellulose column (30 x 2.5 cm) pre-equilibrated in 10 mM potassium phosphate buffer, pH6.0. The column was washed with the same buffer (300 ml), to remove non-bound material, until the optical density at 280 nm returned to base level. The proteins

were then eluted from the column with a linear gradient from 10 mM potassium phosphate buffer, pH6.0 (600 ml) to 500 mM potassium phosphate buffer, pH6.0 (600 ml) at a rate of 70 ml/h. Fractions (14 ml) were collected and the optical density at 280 nm measured. Solid ammonium sulphate was slowly added to the major peaks to give saturated solutions, which were then stirred for 16 h at 4°C. The precipitates were collected by centrifugation (30,000 g, 10 min, 4°C), dissolved in distilled water and dialysed for 16 h at 4°C against distilled water (3 x 4 L). The non-dialysable material was lyophilized and stored at 4°C. The protein content of each peak was determined by the method of Lowry et al. (1951). The activity of the α-toxin in each peak was assayed by competition with [^{125}I]α-BGT for binding to AChR as follows:- Purified Torpedo AChR (100 µl, 0.3 pmol) in toxin binding assay buffer was pre-incubated for 30 min at 23°C with the test sample (20 µl). [^{125}I]α-BGT (50 µl, 0.3 pmol) in toxin binding assay buffer was added and the mixture incubated for a further 60 min at 23°C. The amount of [^{125}I]α-BGT bound was determined by the DEAE-cellulose filtration assay (see "Methods" section 7.2). The samples assayed for α-toxin activity were serially diluted until less than 50% inhibition of [^{125}I]α-BGT binding was observed.

3. Preparation of the α-toxin affinity resin.

The α-toxin purified from Naja naja siamensis crude venom was coupled to activated Sepharose 4B (March et al. 1974) following the method of Lindstrom et al. (1981). Sepharose 4B (50 ml packed beads) was washed with 0.1 M sodium chloride (1 L), followed by distilled water (500 ml). The beads were re-suspended in cold distilled water (100 ml) and 2 M sodium carbonate (100 ml) added and stirred over ice-water to 4°C. Cyanogen bromide (2.5 g) dissolved in acetonitrile (2.2 ml) was added to the Sepharose 4B solution and stirred for 2 minutes at 4°C. The mixture was rapidly filtered and washed with ice-cold distilled water (500 ml). Purified α-toxin (25 mg) was dissolved in 0.2 M sodium hydrogen carbonate, pH9.4 (100 ml), and the optical density at 280 nm was measured. The Sepharose beads were then added to the α-toxin solution which was then stirred overnight at 4°C. The affinity beads were collected by filtration and the eluant retained for optical density measurement at 280 nm. The beads were then washed with distilled water (400 ml) resuspended in 2 M glycine, pH9.0 (200 ml) and stirred overnight at 4°C to block unreacted groups. The affinity beads were again filtered and washed, sequentially, with 0.1 M acetate buffer, pH4.0, containing 1 M sodium chloride (150 ml) and 0.1 M borate buffer, pH8.0 containing 1 M sodium chloride (150 ml). This washing process was repeated 3 times, after which the beads were equilibrated in 10 mM potassium phosphate buffer, pH7.4, containing 0.1% (v/v) Triton X-100, and stored at 4°C in the presence of 0.02% (w/v) sodium azide. After use, the affinity column was washed with 10 mM potassium phosphate buffer, pH7.4, containing 1 M sodium chloride (300 ml) followed by 10 mM potassium phosphate buffer, pH7.4 (500 ml).

4. Purification of AChR

4.1 Human adult or foetal AChR

AChR from human adult or foetal skeletal muscle was purified essentially by the method of Stephenson et al. (1981). All manipulations were performed at 4°C, and the buffers were freshly prepared in double distilled water. Muscle (300 g) was coarsely chopped and homogenized in a Waring blender at maximum speed for 1 min in 4 volumes of buffer 'A' comprising 0.02 M potassium phosphate buffer, pH7.4, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM benzethonium chloride, 2 mM benzamidine hydrochloride, bacitracin (500 μg/ml) and 0.01% (w/v) sodium azide. The homogenate was centrifuged (20,000 g, 60 min, 4°C) and the resulting supernatant was decanted and discarded. The pellet was re-suspended and homogenized as before in 1 volume of buffer 'A' containing additionally 2.5% (v/v) Triton X-100 (extraction buffer), stirred for 3 h at 23°C, and centrifuged (100,000 g, 60 min, 4°C). The resulting supernatant was filtered through glass wool. Varying amounts of this 'crude' muscle extract were retained for use in later assays. The remainder was stirred for 4 h at 23°C with Sepharose 4B (25 ml) coupled to Naja naja siamensis α-toxin. The Sepharose beads were then washed three times alternately on a scintered glass funnel with buffer 'B' (3 x 250 ml) containing 0.5 M sodium chloride and buffer 'B' alone (3 x 250 ml); where buffer 'B' comprised 10 mM potassium phosphate buffer, pH7.4, containing 1 mM EDTA, 0.1 mM benzethonium chloride, 0.1 mM PMSF, 0.02% (w/v) sodium azide and 0.1% (v/v) Triton X-100. The receptor protein was eluted from the Sepharose beads by stirring with 0.5 M carbachol in buffer 'B' (50 ml) for 16 h at 4°C, followed by washing with buffer 'B' alone (50 ml). The eluate was dialyzed against

buffer 'B' (5 L) for 3 h at 4°C, and residual carbachol was removed by passage through a column (5 x 1 cm) of DEAE-cellulose, pre-equilibrated in buffer 'B'. The column was washed, at 4°C, with buffer 'B' (2 L) and the receptor protein eluted with 0.5 M sodium chloride in buffer 'B' (20 ml). 20 x 1 ml fractions were collected, and aliquots of these fractions assayed for [^{125}I]α-BGT binding activity (see "Methods" section 7.2). The peak fractions containing the receptor protein were pooled, dialyzed against buffer 'B' and stored at 4°C.

4.2 Junctional and extra-junctional rat AChR.

Diaphragms from 3-month old female Wistar rats were unilaterally denervated under diethyl ether anaesthesia by section of the left phrenic nerve within the pleural cavity. After 18 days the rats were killed by cervical dislocation and their diaphragms removed. The diaphragms were dissected into denervated and innervated halves. The denervated hemi-diaphragms were finely chopped and homogenized in 4 volumes of homogenization buffer (which comprised 0.02 M potassium phosphate buffer, pH7.4, 1 mM EDTA, 0.1 mM PMSF and 0.01% (w/v) sodium azide) in a Sorvall Omnimix for 1 min at full speed. The homogenate was centrifuged (20,000 g, 45 min, 4°C) and the resulting supernatant decanted and discarded. The pellet was re-homogenized as before in 1 volume of homogenization buffer containing additionally 2.5% (v/v) Triton X-100 (extraction buffer), stirred for 2 h at 23°C and centrifuged (100,000 g, 60 min, 4°C). The resulting supernatant gave a 'crude' detergent extract of extra-junctional AChR. The end-plate regions from innervated rat diaphragms were dissected out and treated as above, giving a 'crude' extract of junctional AChR.

4.3 Torpedo marmorata AChR

AChR was purified from Torpedo marmorata as described by Wonnacott et al. (1980).

5. Purification of human immunoglobulin G (IgG)

IgG was prepared from normal and myasthenic sera by the method of Stevenson and Dorrington (1970). A solution of saturated ammonium sulphate (6 ml, saturated in 0.2 M TRIS-HCl, pH8.0) was added dropwise to normal or myasthenic serum (10 ml) with stirring at 4°C. The solution was stirred for a further 30 min at 4°C and the precipitate sedimented by centrifugation (500 g, 15 min). The precipitate was dissolved in 0.03 M potassium phosphate buffer, pH7.3 (10 ml) and dialysed overnight at 4°C against the same buffer (1 L). The non-dialysable material was applied to a column (13.2 x 2.9 cm) of DE-52 cellulose pre-equilibrated with 0.03 M potassium phosphate buffer, pH7.3. The column was eluted with the same buffer and the absorbance at 280nm of the eluant containing IgG was monitored. Purified IgGs so obtained were stored at -20°C in the presence of 0.02% (w/v) sodium azide.

6. Preparation of goat anti-(human IgG) antiserum.

Normal human IgG (3.0 ml, 1.5 mg/ml) was emulsified with Freund's complete adjuvant (3.0 ml) and injected intra-muscularly at one site in each hind leg of a goat. Repeated injections of IgG in Freund's incomplete adjuvant were performed at 3-weekly intervals. A blood sample (10 ml) was taken from the goat before each injection to allow the determination of anti-(human IgG) antibodies. When an adequate titre was reached, the goat was bled out. The blood was allowed to clot at 4°C overnight, and then centrifuged (5000 g, 20

min, 4°C). The serum was collected and stored at -20°C in the presence of sodium azide. An adequate goat anti-(human IgG) antibody titre was one where less than 100 µl of antiserum was needed to effect total precipitation of [^{125}I]α-BGT-labelled-AChR-antibody complex obtained by incubation of a fixed volume (5 µl) of myasthenic serum with a constant amount of [^{125}I]α-BGT labelled adult human AChR in the presence and absence of benzoquinonium chloride. The details of the assay method are given in "Methods" section 8.

7. Measurement of [^{125}I]α-BGT binding

7.1 Ammonium sulphate precipitation method

AChR in 'crude' detergent extracts of muscle was determined by a method adapted from that of Meunier et al. (1972). Triplicate samples of the receptor extract (100 µl) were incubated with 10 - 15 nM [^{125}I]α-BGT in extraction buffer (50 µl) for 45 min at 23°C before the addition of saturated ammonium sulphate to give 40% saturation overall, and further incubation for 16 h at 4°C. The resulting precipitates were collected on Whatman GFC glass fibre filter discs, washed with 40% saturated ammonium sulphate solution (3 x 1 ml) by vacuum filtration on a Millipore filter unit, and counted for radioactivity. Sufficient excess of [^{125}I]α-BGT was ensured by repetition of the assay using serial 2-fold dilutions of the receptor extract in extraction buffer, when a linear relationship between dilution number and precipitated radioactivity should be obtained. Specific binding of [^{125}I]α-BGT was blocked in parallel incubations performed as above, but containing additionally 1 mM benzoquinonium chloride. Subtraction of the counts so obtained gave specifically bound radioactivity in the test sample. One picomole of

AChR is defined as the amount of receptor that binds one picomole of [^{125}I]α-BGT under saturating conditions. This allows the calculation of the amount of receptor present as:-

$$\underset{(\text{pmol/ml})}{\text{AChR}} = \frac{\text{cpm(sample)} - \text{cpm(blank)}}{\text{cpm(total)}} \times \underset{\text{input}}{\text{pmol toxin}} \times \underset{\text{dilution}}{\text{sample}} \times 10$$

7.2 DEAE-cellulose filtration method

Purified AChR was measured using a DEAE-cellulose filtration assay similar to that described by Schmidt and Raftery (1973). Duplicate serial dilutions of receptor sample (final volume 100 µl) in toxin binding assay buffer (which comprised 10 mM potassium phosphate buffer, pH7.4, containing 0.1% (w/v) BSA, 1% (v/v) Triton X-100 and 0.01% (w/v) sodium azide) were incubated with [^{125}I]α-BGT (50 µl, 0.2 pmol) for 90 min at 23^{o}C (or overnight at 4^{o}C). Each sample was then applied to 2 DEAE-81 cellulose filter discs (diameter 24 mm), pre-moistened with toxin binding assay buffer, left to stand for 2 min, followed by vacuum filtration in a Millipore filter unit. The filters were washed (3 x 1 ml) with toxin binding assay buffer and counted for radioactivity. Specific binding was blocked in parallel incubations performed as above, but containing additionally 1 mM benzoquinonium chloride. Determination of specifically bound [^{125}I]α-BGT allows the calculation of pmol AChR/ml as described in the previous section.

7.3 Immunoprecipitation method

Purified AChR and detergent extracts of AChR were also determined by an immunoprecipitation method involving the addition of a large molar excess of myasthenic serum (5 µl) to [^{125}I] α-BGT-labelled AChR (100 µl), followed by precipitation with goat

anti-(human IgG) antiserum. The conditions of incubation and relevant controls for the non-specific precipitation of radioactivity are the same as those for the radioimmunoassay procedure described below. Sera from MG patients, when used in excess, vary in their ability to precipitate all available [^{125}I] α-BGT labelled AChR complex (see "Results" sections 4.2 & 4.3), therefore care must be taken to use serum from a selected individual if values are to be consistent and comparable with those of other methods. The greatest precipitation of complex was consistently obtained by serum from one particular patient and represented 95 - 100% of that precipitated by ammonium sulphate, and it was therefore this serum that was routinely used. The AChR concentration was calculated from the specifically precipitated radioactivity and expressed as pmoles [^{125}I]α-BGT binding sites/ml of extract.

8. Radioimmunoassay of anti-(AChR) antibodies not directed at the α-BGT binding site.

A radioimmunoassay procedure similar to that previously described (Lindstrom at el., 1976a; Carter et al., 1981) and used in the clinical assay of sera from myasthenic patients was used to examine the binding of anti-(AChR) antibodies to crude and purified human adult and foetal antigens. A final concentration of crude or purified human adult or foetal AChR of greater than 0.5 nM of specific toxin binding sites was labelled by incubation with a 10-fold molar excess of [^{125}I]α-BGT for 45 min at 22^{o}C. The specific binding of [^{125}I]α-BGT was blocked in parallel incubations which contained additionally 1 mM benzoquinonium chloride. Duplicate samples (100 μl) of the resulting solutions were incubated with myasthenic serum or IgG (5 μl), appropriately diluted with normal

human serum or IgG, respectively, for 2 h at 22°C (or 16 h at 4°C). The labelled AChR-antibody complex was precipitated by the addition of goat anti-(human IgG) antiserum (65 - 100 µl) and incubation for 16 h at 4°C (or 2 h at 22°C). The resulting precipitates were collected by centrifugation (3000 g, 10 min, 4°C) and the pellets washed twice with 10 mM potassium phosphate buffer, pH7.4, containing 0.15 M sodium chloride, 1% (v/v) Triton X-100 and 0.1% (w/v) sodium azide (radioimmunoassay buffer), by alternate resuspension and centrifugation, and counted for radioactivity. Subtraction of the counts obtained in the presence of benzoquinonium chloride from counts obtained in the absence of benzoquinonium chloride gave specifically bound radioactivity in the test sample, which can then be related to the concentration of α-BGT binding sites in the AChR extract, and therefore the antibody titre is expressed as moles of specific α-BGT binding sites precipitated per litre of serum. For each patient, maximal formation of [^{125}I] α-BGT-AChR-antibody complex was ensured by repetition of the assay using serial 2-fold dilutions of sera or IgG in order to obtain a linear relationship between the volume of undiluted serum or IgG and precipitated radioactivity.

9. Radioimmunoassay for rabbit anti-(α-BGT) antibodies.

Duplicate serial 2-fold dilutions of rabbit anti-(α-BGT) antiserum (5 µl) in normal rabbit serum were incubated with [^{125}I] α-BGT (0.5 pmol, 50 µl) for 2 h at 23°C. The specific binding of [^{125}I]α-BGT was blocked in parallel incubations which contained additionally a 1000-fold molar excess of unlabelled α-BGT (25 µl). Toxin-antibody complexes were precipitated by the addition of goat anti-(rabbit IgG) antiserum (30 µl) and incubation for 16 h at 4°C.

The resulting precipitates were collected by centrifugation (3000 g, 10 min, 4°C) and the pellets washed twice with radioimmunoassay buffer (see "Methods" section 8) by alternate resuspension and centrifugation, and counted for radioactivity. Subtraction of counts obtained in the presence of unlabelled α-BGT from counts obtained in the absence of unlabelled α-BGT gave specifically bound [^{125}I]α-BGT in the test sample. The antibody titre was then expressed as moles of specific α-BGT binding sites precipitated per litre of serum.

10. Protein measurements

Protein was determined by the method of Lowry et al. (1951) using BSA as standard. For samples containing Triton X-100, the sodium carbonate reagent contained additionally 5% (w/v) sodium dodecyl sulphate (Wang & Smith, 1975).

11. Displacement of [^{125}I]α-BGT from [^{125}I]α-BGT labelled AChR complexes by anti-(AChR) antibodies from myasthenic sera

The time course for displacement of [^{125}I]α-BGT from [^{125}I] α-BGT labelled AChR complexes by anti-(AChR) antibodies was followed. AChR (final concentration 0.25 nM) was incubated in the presence and absence of a large molar excess of benzoquinonium chloride (final concentration 2.5 mM) with [^{125}I]α-BGT (final concentration 0.5 nM) in extraction buffer (see "Methods" Section 4.1) for 16 h at 4°C. In some cases a 10-fold molar excess of [^{125}I]α-BGT was used, in which case incubation was for 90 min at 23°C, followed by gel filtration on a G-75 Sephadex column (1.2 x 16 cm) to remove non-bound [^{125}I] α-BGT. The column was pre-equilibrated and eluted in extraction buffer, fractions (25 x 1 ml) were collected and aliquots (50 µl)

counted for radioactivity. The void volume peak containing [^{125}I] α-BGT labelled AChR was pooled and used as described below.

Duplicate samples of [^{125}I]α-BGT-AChR (100 µl) labelled in the presence and absence of benzoquinonium chloride were incubated with buffer, unlabelled α-BGT, normal human serum or myasthenic serum (5 - 150 µl) for various times at 23°C, after which the reaction was stopped by one of two methods:-

(i) ion-exchange filtration on DEAE-cellulose filter discs - see "Methods" section 7.2.

(ii) gel-filtration on G-75 Sephadex. Samples were applied to a column (1.2 x 16 cm) of G-75 Sephadex, pre-equilibrated in extraction buffer. The column was eluted with the same buffer, fractions (0.5 ml) collected and aliquots (10 µl) counted for radioactivity.

12. Binding properties of detergent-solubilized human adult and foetal AChRs with [^{125}I]α-BGT

Throughout these experiments unfractionated [^{125}I]α-BGT was used (see "Methods" section 1.1), and "crude" detergent extracts of adult and foetal muscle AChR (see "Methods" section 4.1)

12.1 Determination of the association constant (K_{on})

The rate of association between detergent-solubilized adult or foetal AChR and [^{125}I]α-BGT was determined from the initial progress curves of the reaction at increasing [^{125}I]α-BGT concentrations. AChR (0.5 nM, 100 µl) in toxin binding assay buffer (see "Methods" section 7.2) was incubated with a range of [^{125}I] α-BGT concentrations (2.8 - 16.8 nM, 50 µl) in the presence and absence of unlabelled α-BGT (5 µM, 50 µl) for varying times upto 90

min at 23°C. The amount of [^{125}I]α-BGT bound was measured by the DEAE-cellulose filtration assay, as described in the "Methods" section 7.2.

12.2 Determination of the dissociation constant (K_{off})

The dissociation of [^{125}I]α-BGT-labelled AChR complexes was studied by allowing the binding of [^{125}I]α-BGT and AChR to reach equilibrium, after which the rebinding of [^{125}I]α-BGT was prevented and the amount of [^{125}I]α-BGT-AChR complex determined at various times thereafter. Detergent extracts of adult or foetal AChR (0.5 nM, 100 µl) in toxin binding assay buffer were incubated with [^{125}I] α-BGT (5 nM, 50 µl) for 90 min at 23°C. A large molar excess of unlabelled α-BGT (5 µM, 10 µl) was then added, and the time course of replacement of [^{125}I]α-BGT by unlabelled α-BGT was followed, using the DEAE-cellulose filtration assay (see "Methods" section 7.2. Samples without the addition of cold α-BGT were assayed in parallel to correct for any changes in binding over this time period.

12.3 Saturation experiments

Detergent extracts of adult or foetal AChR (0.5 nM, 100 µl) in extraction buffer (see "Methods" Section 4.1) were incubated with a range of [^{125}I]α-BGT concentrations (0.085 - 51 nM, 50 µl; giving 0.025 - 15 nM final concentrations) in the presence and absence of unlabelled α-BGT (12.75 µM, 20 µl; giving 1.5 µM final concentration) for 15 min at 23°C. Saturated ammonium sulphate was added to give a final concentration of 40% saturation overall, and samples were then incubated for 16 h at 4°C. The resulting precipitates were collected and counted for radioactivity as described in "Methods" section 7.1.

13. Immunohistochemistry

13.1 Gelatinization of microscope slides

Microscope slides were cleaned with 50% (v/v) alcohol. Gelatin solution was made up freshly by adding 0.5% (w/v) gelatin and 0.05% (w/v) chrome alum to distilled water, heating to 80°C and then filtering through Whatman filter paper. Slides were dipped into the gelatin solution and allowed to drain and dry at room temperature.

13.2 Cryostat sectioning of tissue material

Small pieces of human adult or foetal limb muscle were dissected and mounted onto cryostat chucks sitting in dry ice using "Tissue Tek" mounting fluid, placed in the cryostat and allowed to equilibrate to cutting temperature (range -15 - -25°C) within the cryostat chamber. Serial transverse sections were taken at 20 μM, collected onto gelatinized slides (see "Methods" section 13.1), dried at room temperature and then stored in sealed boxes at -20°C until required (less than 1 month), unless used immediately.

13.3 Immunohistochemical staining using the Peroxidase anti-Peroxidase (PAP) technique.

Slides with human adult or foetal muscle tissue were immersed and incubated in one of the following solutions, made up in 50 mM potassium phosphate buffer, pH7.4:-

a) buffer alone (60 min at 23°C)

b) 0.5 nM α-BGT alone in buffer (60 min at 23°C)

c) 1 mM curare in buffer (30 min at 23°C) followed by 0.5 nM α-BGT (30 min at 23°C).

The tissue sections were then washed in 150 mM potassium

phosphate buffer, pH7.4 (2 x 5 min at 23°C) and fixed in either 4% (w/v) paraformaldehyde in 0. 1 M potassium phosphate buffer, pH7.4 for 30 min at 4°C or in glacial acetic acid:ethanol (5:95 (v/v)) mixture for 10 min at -20°C, washed thoroughly in 0.1 M potassium phosphate buffer, pH7.4 (2 x 30 min at 23°C or PBS containing 0.003% (w/v) phenol red as indicator (3 x 5 min at 23°C) respectively and incubated with non-absorbed or absorbed rabbit anti-(α-BGT) antiserum (see "Methods" section 13.4) for 60 min at 23°C or overnight at 4°C (final dilution 1:200 in PBS, pH7. 4, containing 0.1% (v/v) Triton X-100; buffer 'A'), then washed in buffer 'A' alone (2 x 10 min at 23°C). The slides were then incubated with sheep anti-(rabbit IgG) antiserum for 30 min at 37°C (final dilution 1:10 in PBS containing 0.1% (v/v) Triton X-100 and 1% (v/v) normal sheep serum; buffer 'B'), followed by washing in buffer 'B' alone (2 x 10 min at 23°C. Sections were then incubated with 'PAP' complex (final dilution 1:50 in buffer 'B') for 30 min at 37°C, and further washed in buffer 'B' alone (2 x 10 min at 23°C) followed by a final wash in 0.05 M TRIS buffer, pH7.6 (15 min at 23°C). The tissue sections were stained with 0.0125% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M TRIS buffer, pH7.6 containing 0.0125% (v/v) hydrogen peroxide for 2 - 20 min at 23°C until colour development was complete, after which they were washed in 0.05 M TRIS buffer, pH7.6 (3 x 5 min at 23°C) followed by dehydration in a series of alcohols:- 70% (v/v) ethanol (2 min at 23°C), 95% (v/v) ethanol (2 min at 23°C), absolute ethanol (2 x 2 min at 23°C) and finally xylene (16 h at 23°C). The slides were embedded and coverslipped in DPX mounting medium and left to harden for 7 days.

13.4 Absorption of rabbit anti-(α-BGT) antiserum

13.4 (i) Using normal human red blood cells.

Normal human peripheral blood (10 ml) was centrifuged (200 g, 10 min, 23°C) and the serum discarded. The red cells were washed 3 times in PBS by alternate resuspension and centrifugation. Packed red cells were incubated with heat-inactivated rabbit anti-(α-BGT) antiserum (1:1 (v/v)) for 2 x 30 min at 23°C, followed by centrifugation, after which the absorbed antiserum was removed and stored at 4°C in the presence of sodium azide.

13.4 (ii) Using human adult and foetal muscle homogenate.

Human adult muscle (25 g) and human foetal muscle (25 g) were coarsely chopped and homogenized together in 4 volumes of 10 mM potassium phosphate buffer, pH7.4, containing 1 mM EDTA in a Sorvall Omnimix at full speed for 1 min. The homogenate was centrifuged (20,000 g, 45 min, 4°C) and the resulting supernatant decanted and discarded. The pellet was re-homogenized and centrifuged as above twice more. After the final centrifugation, the pellet was re-suspended in 1 volume of rabbit anti-(α-BGT) antiserum (diluted 1:20 in 10 mM potassium phosphate buffer, pH7.4, containing 1 mM EDTA) and left for 2 h at 4°C, followed by centrifugation (100,000 g, 60 min, 4°C). The supernatant was assayed for anti-(α-BGT) activity (see "Methods" section 9) and stored at 4°C in the presence of sodium azide.

13.5 Fluorescein-conjugated α-BGT staining of teased muscle fibres

Human adult or foetal skeletal muscle was teased into fibres under a Swift dissecting microscope and washed in 0.1 M HEPES buffer, pH7.4. Bundles of fibres were then dried onto gelatinised slides. The slides were then pre-incubated in the presence and absence of a large molar excess (10^{-6} M) of unlabelled α-BGT in buffer 'A' (which comprised 0.1 M HEPES buffer, pH7.4, containing 1% (v/v) heat inactivated normal human serum) for 60 min at 23°C, washed in buffer 'A' (2 x 10 min) and incubated with fluorescein-conjugated α-BGT (1 - 10 μg/ml) in buffer 'A' for 90 min at 23°C. Non-bound material was removed by washing in buffer 'A' (5 x 10 min), after which the sections were fixed in 4% (w/v) paraformaldehyde in buffer 'A' for 30 min at 4°C, then washed in buffer 'A' (2 x 5 min) followed by PBS, pH7.4 (1 x 15 min). The muscle fibres were then counterstained in 0.01% (w/v) methyl green in PBS, pH7.4 for 5 min at 23°C, washed in PBS, pH7.4 (3 x 5 min), mounted in glycerol and viewed immediately.

13.6 Acetylcholinesterase staining of teased muscle fibres

Human adult or foetal skeletal muscle fibres were teased and slides prepared as described in the previous section. The slides were incubated for 2 h at 23°C in staining solution (which comprised 15% (w/v) sodium sulphate, 70 mM maleic acid, 20 mM glycine, 4 mM $CuSO_4.5H_2O$, adjusted to pH6.5 (this solution is stable for up to 2 weeks at 23°C), followed by the addition of 0.02% (w/v) acetylthiocholine iodide just prior to use). Control slides were incubated in the staining solution

which contained additionally 100 μM neostigmine. The slides were then washed with PBS, pH7.4 (3 x 5 min), after which the stain was developed by incubation in dithio-oxamide (prepared by making a saturated solution and then filtering through Whatman No. 1 paper). When a dark brown stain had developed (approximately 3 min) the slides were washed extensively in PBS, pH7.4 (5 x 3 min), mounted in glycerol and viewed immediately.

RESULTS

1. Iodination and characterization of α-BGT

1.1 Preparation of [^{125}I]-labelled α-BGT

α-BGT was iodinated by the chloramine-T method (see "Methods" section 1.1). A typical elution profile from the G-25 Sephadex column is shown in Figure 7, where it can be seen that approximately 90% of the [^{125}I] was incorporated into the protein (range 68 - 97%; mean ± SE (n) = 88 ± 1.8% (20)). The specific radioactivity was in the range 590 - 780 Ci/mmol (0.074 - 0.098 μCi/μg), with mean value ± SE (n) of 710 ± 15 (20) Ci/mmol (0.089 ± 0.0018 (20) μCi/μg).

1.2 Fractionation of [^{125}I]-labelled α-BGT species.

α-BGT contains 2 tyrosine residues which may become iodinated, giving rise to a mixture of mono- and di-iodinated species (Vogel et al., 1972). These were separated by ion-exchange chromatography on CM-Sephadex C-50 (see "Methods" section 1.2). A typical elution profile is shown in Figure 8. The first peak, eluted during loading and washing, was free [^{125}I], the second peak, eluted with 10 mM NaCl, was di-iodinated α-BGT, and the third peak, eluted with 100 mM NaCl was mono-iodinated α-BGT (Vogel et al., 1972).

1.3 Determination of the concentration of iodinated α-BGT species by dilution assay.

The concentration of iodinated α-BGT species obtained from fractionation on CM-Sephadex C-50 was determined by dilution assay (see "Methods" section 1.3). The specific binding of each iodinated α-BGT species to Torpedo AChR at 4 different concentrations of

Figure 7. Gel filtration of [^{125}I]α-BGT on Sephadex G-25

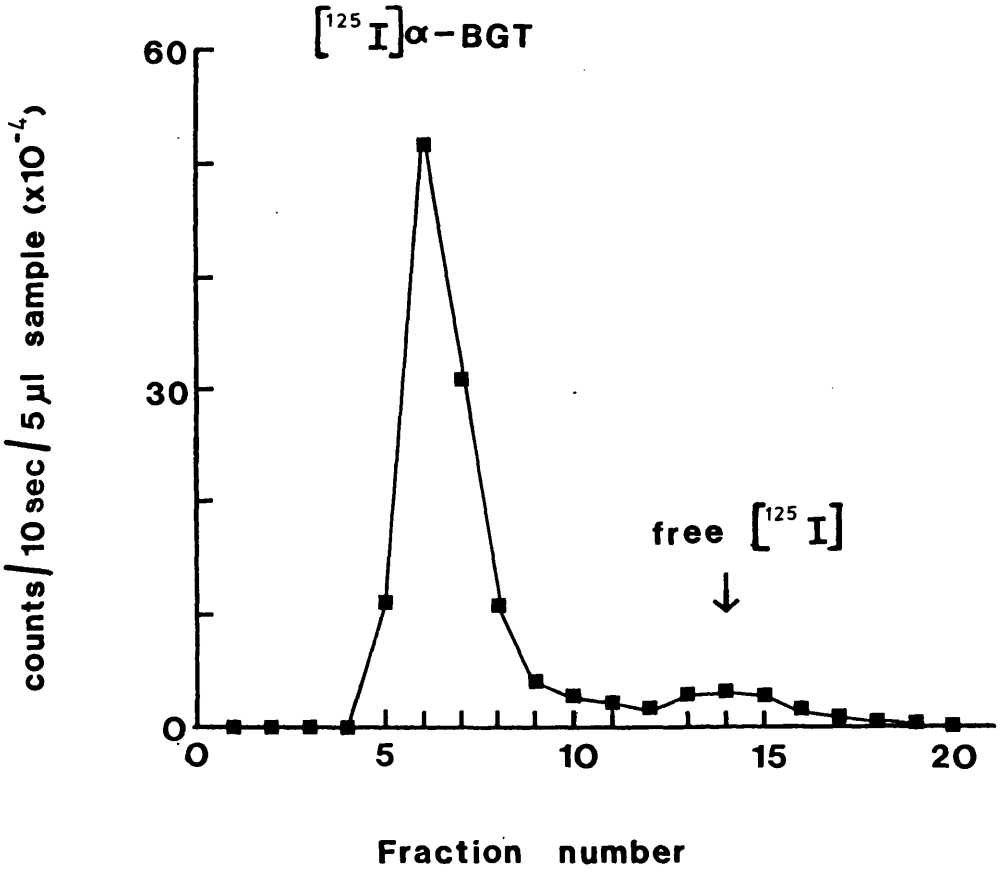
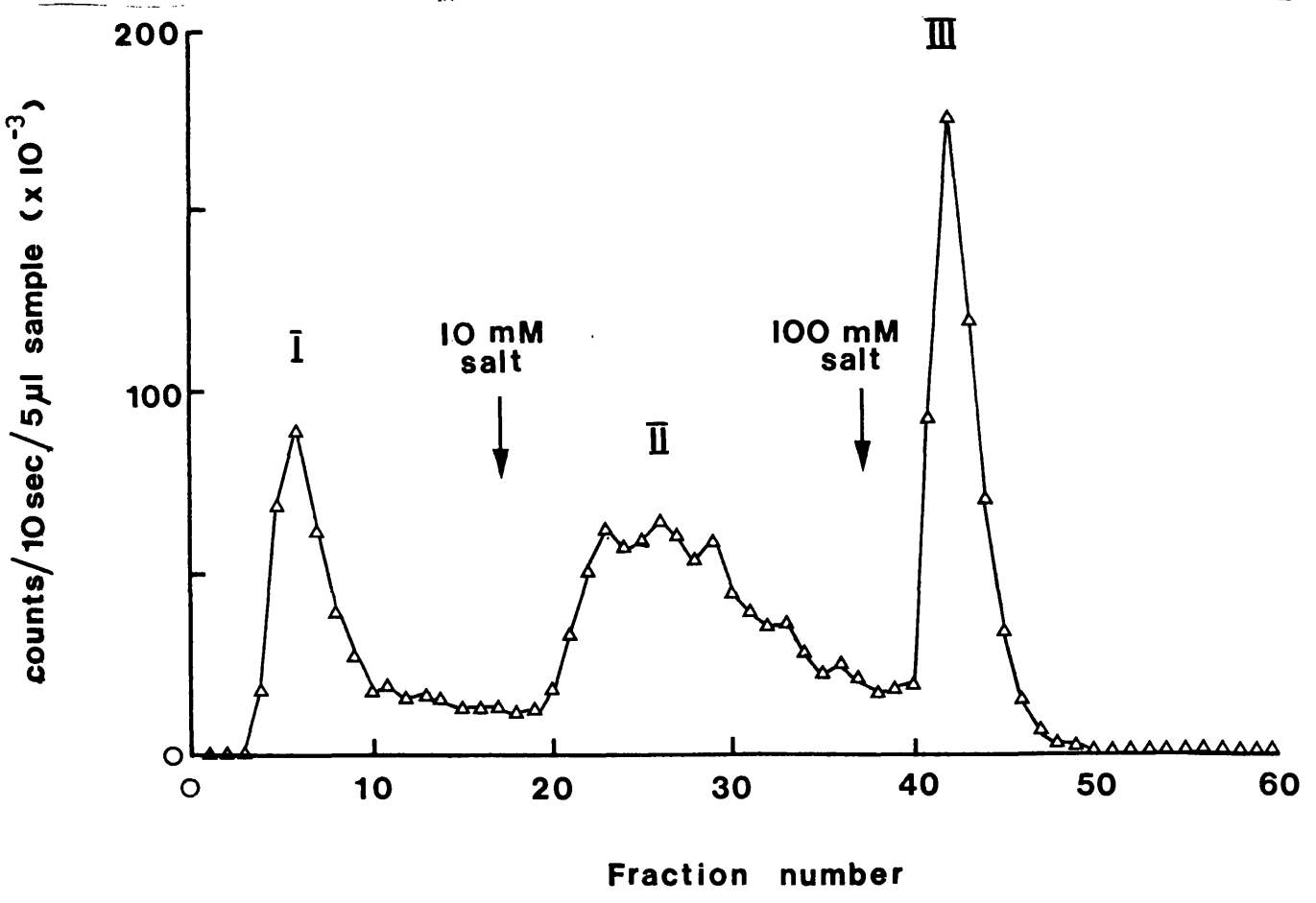


Figure 8. Fractionation of iodinated α-BGT species by ion-exchange chromatography on CM-Sephadex C-50



unlabelled α-BGT was plotted against the concentration of Torpedo AChR used. The reciprocals of the slopes of the graphs so obtained were re-plotted against the concentrations of unlabelled α-BGT used. The intercept on the x-axis then gives the amount of iodinated α-BGT species present in the test sample used. Typical curves obtained are shown in Figure 9. Values obtained for unfractionated [^{125}I]α-BGT by this method agreed well with those obtained in section 1.1.

1.4 Determination of the biological activity of radiolabelled α-BGT species.

Biological activities of radiolabelled α-BGT species were assessed by their ability to bind to a large molar excess of Torpedo AChR. Toxin-receptor complexes were separated from unbound species by ion-exchange chromatography on DEAE-cellulose filter discs or by gel filtration on a Sephadex G-50 column (see "Methods" section 1.4). Biological activities of unfractionated [^{125}I]α-BGT were routinely assayed by the DEAE-cellulose filtration method and gave mean values ± SE of 57 ± 5% (n = 6). A comparison of the 2 methods was undertaken for various α-BGT species, including [^{3}H]α-BGT, and the results obtained are shown in Figure 10 and Table 1. It can be seen that the biological activities obtained by separation of bound and free species on a gel filtration column were in the order of 90 - 100%, and were consistently higher than the values obtained by ion-exchange chromatography on DEAE-cellulose discs, which gave values of 40 - 50%. Underestimation of toxin binding using DEAE-celluose filter discs was noted during other assays, and this is discussed further in "Results" section 3.2. The biological activity of unfractionated [^{125}I]α-BGT was also assessed by its

Figure 9. Determination of the concentration of iodinated α-BGT species by dilution assay

The concentration of iodinated α-BGT species obtained from fractionation of [^{125}I]α-BGT on CM-Sephadex C-50 was determined by dilution assay (see "Methods" section 1.3).

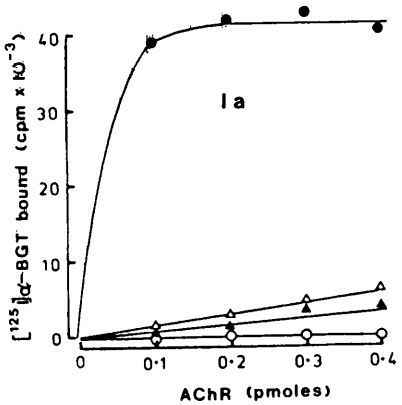
1. Free [^{125}I] (peak 1
2. Di-iodinated α-BGT (peak II
3. Mono-iodinated α-BGT (peak III

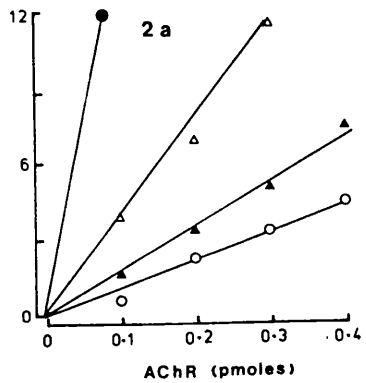
} from CM-Sephadex C-50 column

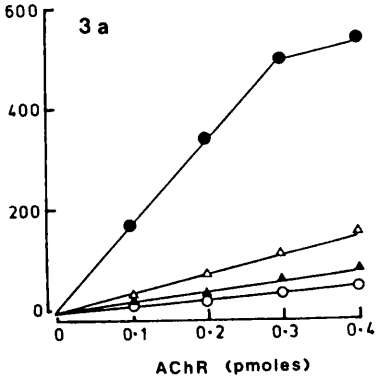
4. Unfractionated [^{125}I]α-BGT

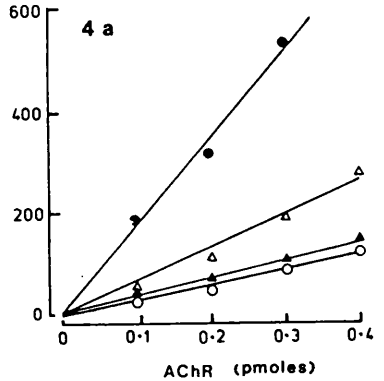
(a) The specific binding of each iodinated α-BGT species to Torpedo AChR at 4 different concentrations of unlabelled α-BGT (● 0 pmoles; △ 4 pmoles; ▲ 8 pmoles; ○ 12 pmoles) was plotted against the concentration of AChR used.

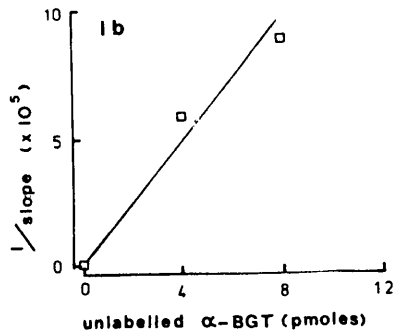
(b) The reciprocals of the slopes of the graphs obtained in (a) were re-plotted against the concentrations of unlabelled α-BGT used. The intercept on the x-axis then gives the amount of iodinated α-BGT species present in the test sample used.

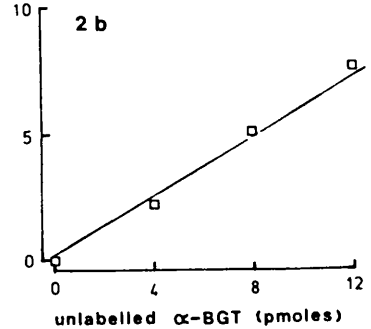

1a
[125I]α-BGT bound (cpm x 10-3)
AChR (pmoles)

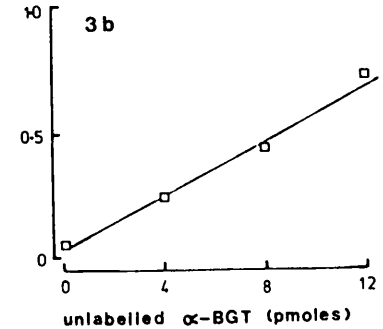

2a
AChR (pmoles)


3a
AChR (pmoles)


4a
AChR (pmoles)


1b
1/slope (x 10^5)
unlabelled α-BGT (pmoles)


2b
unlabelled α-BGT (pmoles)


3b
unlabelled α-BGT (pmoles)

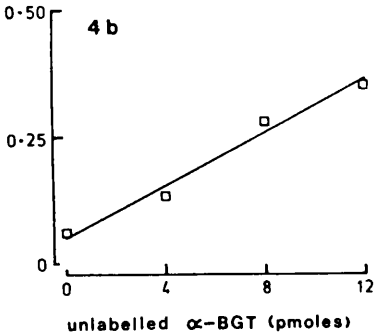

4b
unlabelled α-BGT (pmoles)

Figure 10. To compare biological activites of various radiolabelled α-BGT species by gel filtration an Sephadex G-50 with DEAE-cellulose disc filtration

Biological activities of various radiolabelled α-BGT species were assessed as described in the "Methods" section 1.4, by gel filtration on Sephadex G-50 or by ion-exchange on DEAE-cellulose filter discs.

▲ 1 ml fractions from Sephadex G-50 filtration

△ The same fractions filtered through 2 DEAE-cellulose filter discs and counted for radioactivity

1. Unfractionated [^{125}I]α-BGT alone
2. Unfractionated [^{125}I]α-BGT incubated with a molar excess of *Torpedo* AChR for 90 min before filtration
3. Mono-iodinated α-BGT incubated with a molar excess of *Torpedo* AChR for 90 min before filtration
4. Di-iodinated α-BGT incubated with a molar excess of *Torpedo* AChR for 90 min before filtration
5. [^{3}H]α-BGT alone
6. [^{3}H]α-BGT incubated with a molar excess of *Torpedo* AChR for 90 min before filtration

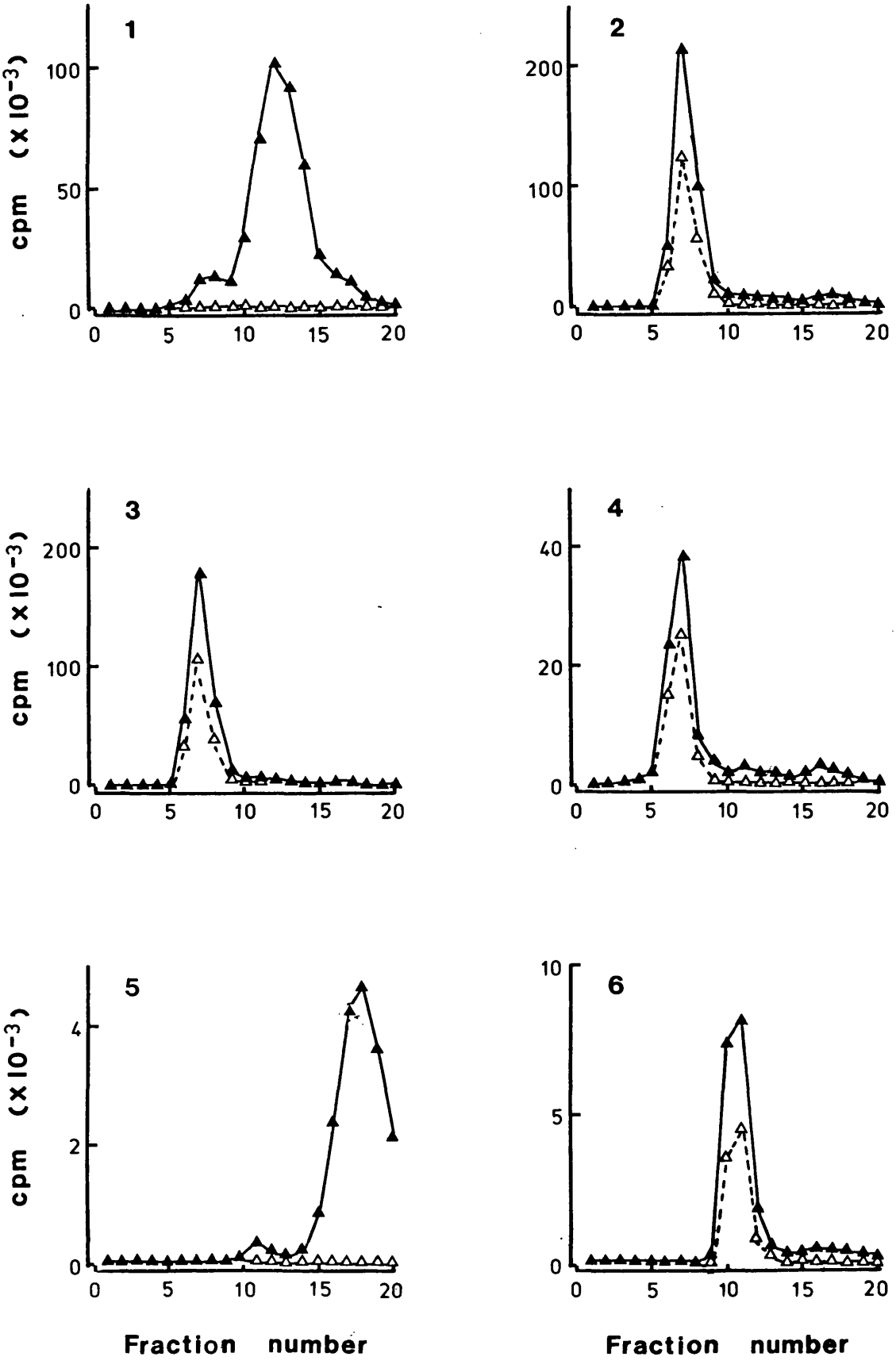

1
2
3
4
5
6
cpm (x10⁻³)
Fraction number

Table 1. A comparison of the biological activities of different radiolabelled α-BGT species measured by gel filtration on Sephadex G-50 and ion-exchange on DEAE-cellulose filter discs.

Radiolabelled α-BGT species	Biological Activity (%)	
	Gel filtration on Sephadex G-50	Ion exchange on DEAE -cellulose filter discs
Unfractionated [^{125}I]α-BGT	92	42
Mono-iodinated α-BGT	95	47
Di-iodinated α-BGT	89	42
[^{3}H]α-BGT	93	43

Each value is the mean of 2 determinations.

ability to bind to a large excess of rabbit anti-(α-BGT) antiserum, the value obtained for the biological activity in this way was 75%.

1.5 Determination of the radiolabelled protein by precipitation with tri-chloroacetic acid.

Table 2 shows a comparison of the values obtained for the percentage precipitation of radiolabelled α-BGT species by 6% (w/v) TCA. Similar values for protein associated iodine were derived for the different species of α-BGT. In particular, unfractionated and mono-iodinated α-BGT appear very similar.

1.6 Comparison of radiolabelled α-BGT species in binding assays.

The binding of different radiolabelled α-BGT species with AChR was compared in the DEAE-cellulose filtration assay, the ammonium sulphate assay and the radioimmunoassay. For the DEAE-cellulose and ammonium sulphate assays 0.2 pmol of each radiolabelled α-BGT species was incubated with serial 2-fold dilutions of purified Torpedo or detergent extracts of adult human AChR, respectively, in the presence and absence of an excess of benzoquinonium chloride, and the assays performed as described (see "Methods" section 7). The radioimmunoassay was performed, using detergent extracts of adult human muscle, as described (see "Methods" section 8), using equivalent amounts of each radiolabelled α-BGT species (5 nM final concentration). Table 3 (a,b,c) shows the results obtained. In the DEAE-cellulose filtration and immunoprecipitation assays, the behaviour of unfractionated [^{125}I] α-BGT, mono-iodinated α-BGT and [^{3}H]α-BGT were comparable; whereas in the ammonium sulphate assay unfractionated and mono-iodinated α-BGT

Table 2. Precipitation of radiolabelled α-BGT by trichloroacetic acid

Radiolabelled α-BGT species	% precipitation by trichloroacetic acid
Unfractionated [^{125}I]α-BGT	86
Mono-iodinated α-BGT	83
Di-iodinated α-BGT	85
[^{3}H]α-BGT	84

Table 3. Comparison of radiolabelled α-BGT species in binding assays

(a) DEAE-cellulose filtration assay

	Radiolabelled α-BGT species			
	Unfractionated [^{125}I]α-BGT	mono-iodo α-BGT	di-iodo α-BGT	[^{3}H] α-BGT
Torpedo AChR (pmol/ml)	3994	3764	2791	3826
% value using unfractionated [^{125}I]α-BGT	100	94	70	96

(b) Ammonium sulphate assay

	Radiolabelled α-BGT species			
	Unfractionated [^{125}I]α-BGT	mono-iodo α-BGT	di-iodo α-BGT	[^{3}H] α-BGT
Human AChR (pmol/ml)	1.94	1.95	1.53	1.47
% value using unfractionated [^{125}I]α-BGT	100	101	79	76

(c) Radioimmunoassay for anti-(AChR) antibodies

Anti-(AChR) antibody titre (nM) obtained using different radiolabelled α-BGT species

Patient Number	Unfractionated [^{125}I]α-BGT	mono-iodo α-BGT	di-iodo α-BGT	[^{3}H] α-BGT
3	221 (-)*	237 (108)	150 (68)	233 (105)
13	7 (-)	8 (122)	4 (62)	7 (97)
6	31 (-)	35 (115)	19 (63)	32 (102)
10	20 (-)	23 (114)	13 (66)	19 (92)
Normal	0 (-)	0 (0)	0 (0)	0 (0)

Values in parentheses are percentage of using unfractionated [^{125}I]α-BGT.

gave similar results, but [^{3}H]α-BGT gave slightly lower values. In all assays di-iodinated α-BGT gave consistently lower binding.

2. Detergent extraction and subsequent partial purification of AChR.

AChR was extracted from different skeletal muscle types using the non-ionic detergent Triton X-100. Some extracts of human muscle were subsequently partially purified by affinity chromatography on a cobra toxin-Sepharose resin and concentration by ion-exchange chromatography on a DEAE-cellulose column. A cocktail of protease inhibitors and anti-bacterial agents was used throughout the procedure in order to minimize degradation by proteolysis. The purification procedure was performed as quickly as possible (within 2 days), and at 4^{o}C, for the same reason.

2.1 Purification of the α-toxin from venom of Naja naja siamensis and preparation of the α-toxin affinity resin.

The α-toxin was purified from Naja naja siamensis crude venom to provide the ligand for the affinity purification of AChR (see "Methods" section 2). The profile of absorbance at 280 nm for elution from the phosphocellulose column is shown in Figure 11. and the results obtained are summarized in Table 4. The strongest -toxin activity was eluted from the phospocellulose column in 3 peaks: D,E & F. Recovery of α-toxin binding activity was 39%, giving an overall 3.4-fold purification. Assuming a MW for α-toxin of 8000, it was 25% pure.

Peaks D,E and F were pooled, and a sample (25 mg) was coupled to cyanogen bromide activated Sepharose 4B (see "Methods" section 3). 76% of the α-toxin was coupled to the resin, indicating a

Figure 11. The elution profile from phosphocellulose ion-exchange chromatography in the purification of the α-toxin from Naja naja siamensis venom

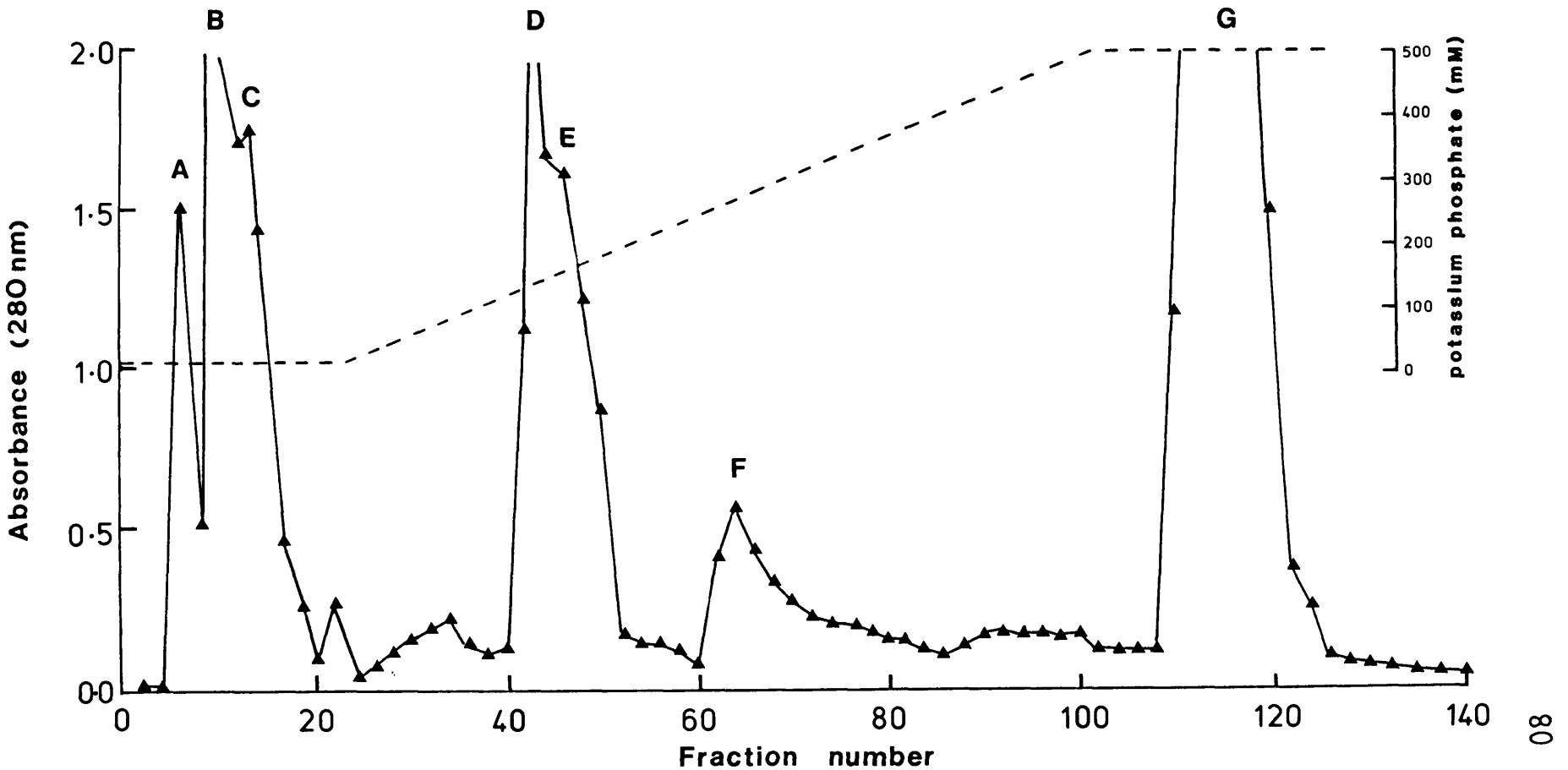


Table 4. Summary of purification of α-toxin from *Naja naja siamensis*

fraction	peak	dilution giving 50% inhibition of [^{125}I]α-BGT binding	total protein (mg)	pmoles α-toxin	specific activity (pmol/mg)
	crude venom	4.9×10^{-7}	1046	1.53×10^{8}	1.46×10^{5}
	A	8.0×10^{-4}	26.52	2.44×10^{5}	9.20×10^{3}
	B	—	18.98	—	—
column	C	—	45.50	—	—
fractions	D	3.0×10^{-6}	120.25	9.25×10^{7}	7.69×10^{5}
	E	1.4×10^{-5}	112.45	3.48×10^{7}	3.09×10^{5}
	F	6.0×10^{-5}	35.20	1.17×10^{7}	3.32×10^{5}
	G	1.0×10^{-4}	798	1.14×10^{7}	1.43×10^{4}
freeze-	D*	2.0×10^{-5}	39.40	1.48×10^{7}	3.76×10^{5}
dried	E*	1.5×10^{-5}	82.70	4.14×10^{7}	5.00×10^{5}
fractions	F*	3.5×10^{-5}	18.20	3.90×10^{6}	2.14×10^{5}

* lyophilized samples at 1 mg/ml in distilled water

— no inhibition of toxin binding activity

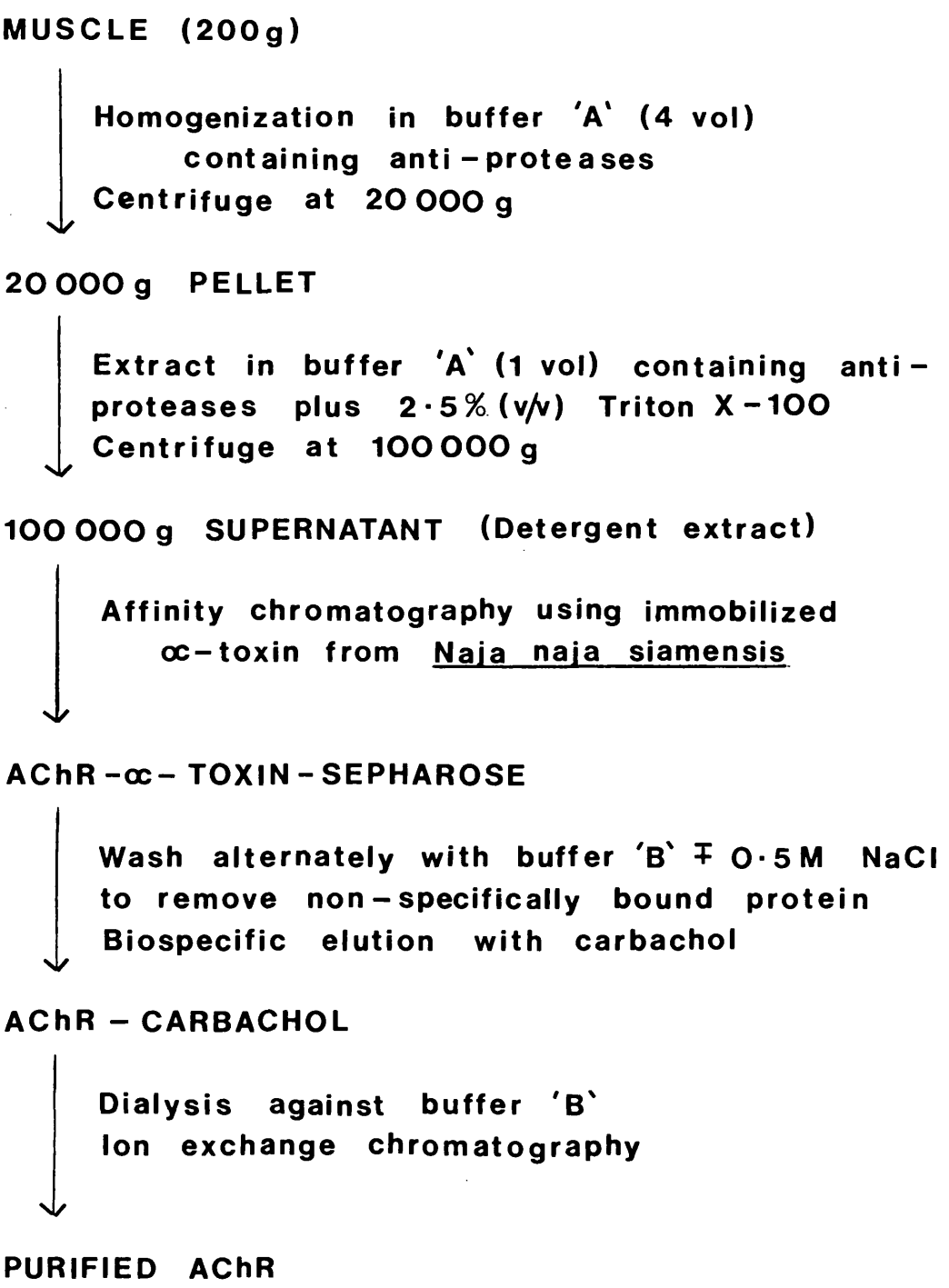
content of 0.38mg α-toxin/ml packed Sepharose 4B beads.

2.2 Human adult and foetal AChR

Detergent extraction of human skeletal muscle gave yields of 0.23 - 2.19 pmoles [^{125}I]α-BGT binding sites per gram of muscle (0.89 ± 0.11, 27 determinations, mean ± SE) for adult tissue; and 0.61 - 1.89 pmoles [^{125}I]α-BGT binding sites per gram of muscle (1.20 ± 0.10, 15 determinations, mean ± SE) for foetal tissue. Storage of tissue for up to 3 months at -80^{o}C did not cause apparent loss of extractable [^{125}I]α-BGT binding sites. In most cases no further purification of the detergent extract was performed but was used in the radioimmunoassay for anti-(AChR) antibodies (see "Methods" section 8).

Purification of AChR by affinity chromatography on immobilized α-Naja toxin, biospecific elution with carbachol and ion-exchange chromatography on DEAE-cellulose was performed as outlined in the "Methods" section 4.1. A flow diagram showing the basic purification scheme is shown in Figure 12. 60 - 90% of the available AChR in "crude" detergent extracts bound to the α-toxin affinity column. Determination of AChR in the diffusate before dialysis was not performed, as measurement of AChR at this stage is complicated by the presence of residual carbachol. Yields of purified AChR were expressed as a percentage of the detergent extractable activity, and represented 6 - 25% recovery. (The total homogenate was too fibrous to allow accurate determination of AChR.) Purified adult AChR preparations showed specific activities of 0.01 - 0.8 nmol/mg protein over 6 preparations; the corresponding values for foetal AChR (0.01 - 0.1 nmol/mg protein) are probably

Figure 12. Flow diagram showing the method for purification of AChR



underestimates due to the rapid loss of [^{125}I]α-BGT binding (see below). The best preparation of purified adult AChR represented a 20000-fold purification compared with the detergent extract. Table 5 shows a typical purification table giving yields and percentage recoveries through the purification procedure. Crude detergent extracts of adult AChR were stable for 2 months at 4^{o}C (Figure 13(a)) without appreciable loss of toxin binding ability; whilst purified preparations of adult AChR were stable for about 1 week at 4^{o}C after which the toxin binding activity decreased rapidly. Crude detergent extracts of foetal AChR stored at 4^{o}C lost 50% of their toxin binding activity in the first 3 weeks after which they remained constant for up to 4 months (Figure 13(b)). Purified preparations of foetal AChR were used immediately as the toxin binding activity was found to disappear within 2 days of purification.

2.3 Junctional and extra-junctional rat AChR

Detergent extraction of rat junctional and extra-junctional AChR from diaphragm muscle gave yields of 0.51 $\pm$ 0.04 (mean $\pm$ SE, n = 5) and 1.21 $\pm$ 0.4 pmol (mean $\pm$ SE, n = 4) [^{125}I]α-BGT binding sites per gram of muscle respectively. The extracts were stored at 4^{o}C for up to 6 months without apparent loss of toxin binding activity.

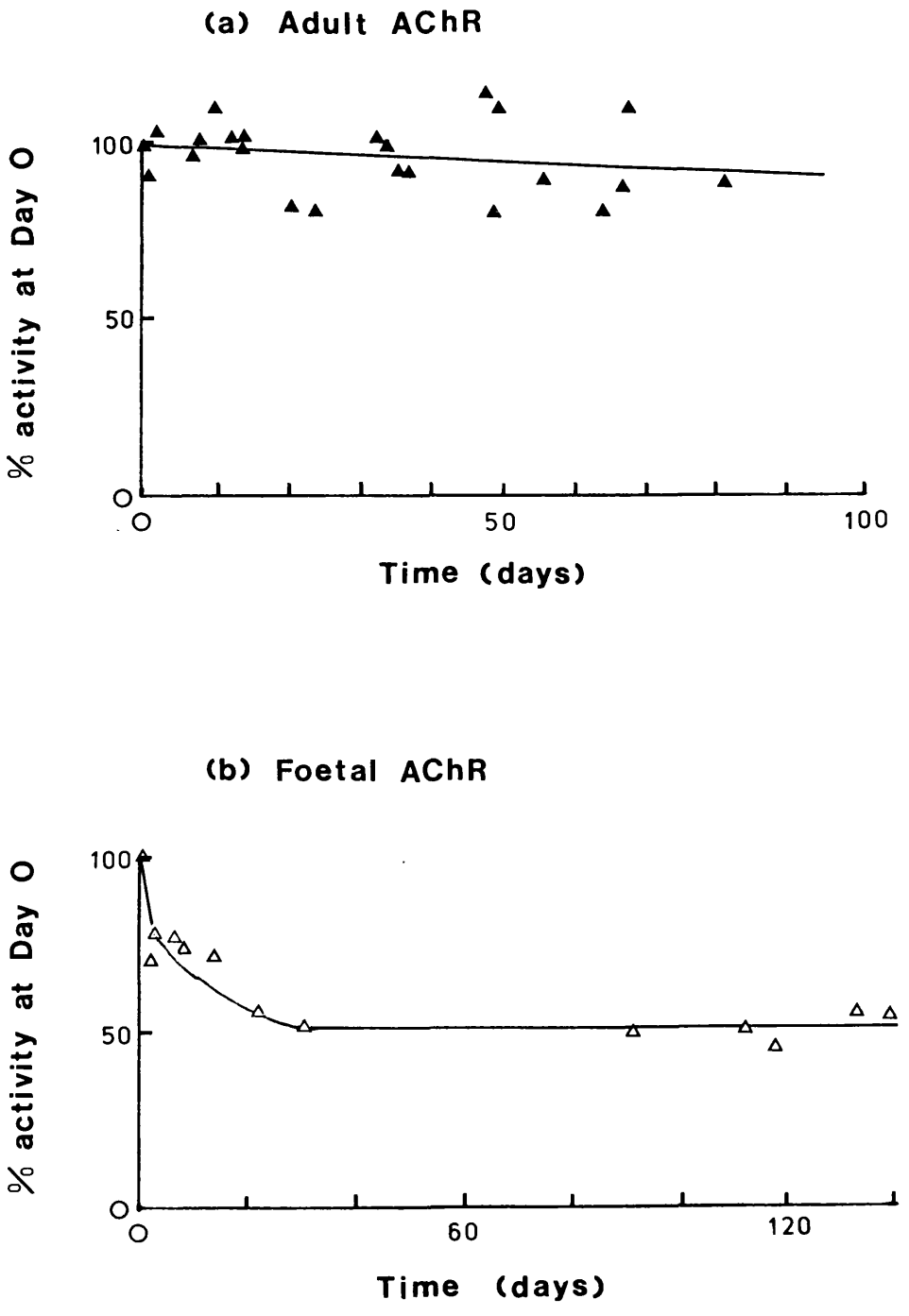
2.4 Torpedo marmorata AChR

The purified AChR had a specific activity of 2.9 nmol specific binding sites/mg protein. Other AChR preparations, with specific activities ranging from 2 - 7 nmol/mg were also used, and were kindly donated by Lindsay Muir.

Table 5. Purification table for recovery of AChR from human adult muscle during the purification procedure

Stage of purification	AChR (pmol)	Protein (mg)	Specific activity (pmol/μg)	Recovery α-toxin binding (%)	Purification factor
Triton X-100 detergent extract	235	1813	0.00013	100	1
Non-bound to affinity resin	25	1694	0.000015	11	
Washes from affinity beads	0	32	0	0	
Non-bound to DEAE-cellulose	0	7	0	0	
Purified adult AChR	35	0.12	0.29	15	2230

Figure 13. Loss of binding activity of detergent extracts of AChR



3. Measurement of [^{125}I]α-BGT binding

AChR was determined by 3 methods (see "Methods" section 7), depending on the nature of the test sample. The DEAE-cellulose filtration assay was used mainly for purified preparations and the ammonium sulphate precipitation assay for "crude" detergent extracts which, because of their high protein content, gave erroneous results in the former assay (see Table 6). The DEAE-cellulose filtration assay was used for kinetic determinations of [^{125}I]α-BGT binding to AChR in detergent extracts of foetal and adult skeletal muscle, where ammonium sulphate was found to be unsuccessful in terminating the binding reaction (see Table 7(a)). For these purposes, high activity extracts were diluted before use and this helped to reduce the non-specific component of binding.

The immunoprecipitation method was used successfully for both detergent extracts and purified preparations.

3.1 Ammonium sulphate precipitation method

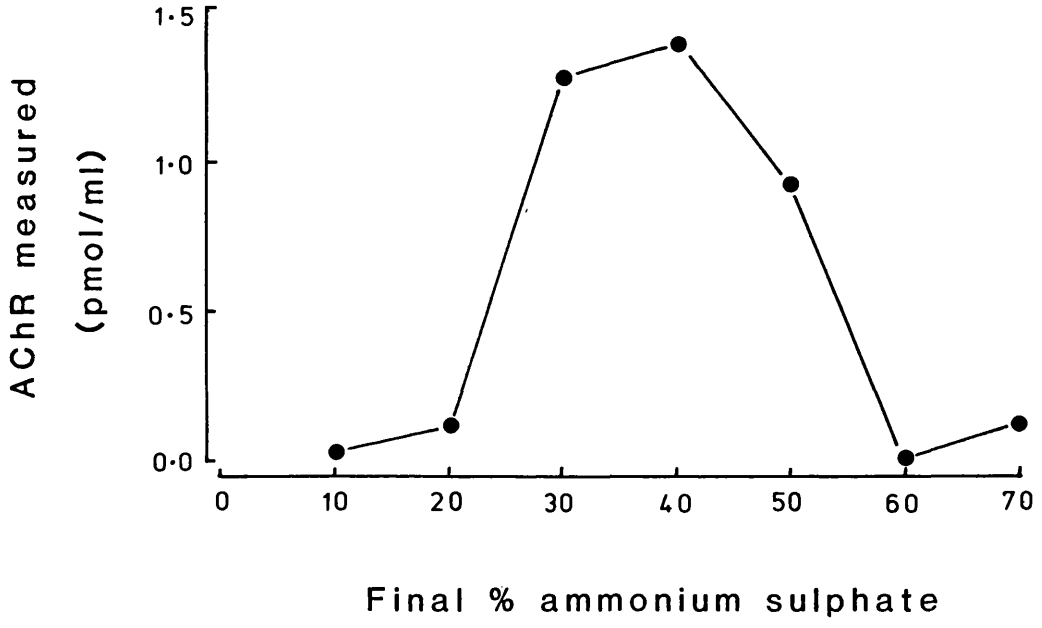
The final percentage saturation of ammonium sulphate required to give maximum precipitation of [^{125}I]α-BGT labelled AChR complex in "crude" detergent extracts was determined by adding increasing amounts of saturated ammonium sulphate to a constant amount of [^{125}I]α-BGT - AChR complex to give final percentage saturations of 10 - 70%. The results are shown in Figure 14 where it can be seen that 40% saturation overall effected maximum precipitation of complex. The assay was linear with protein concentration if excess [^{125}I]α-BGT was used. The contribution of [^{125}I]α-BGT to the assay blank was assessed by incubating increasing amounts of [^{125}I]α-BGT (0 - 3.5 pmol, representing 0 - 20 nM in the assay) with 40% final

Table 6. Comparison of measurement of AChR in detergent extracts by DEAE-cellulose filtration and ammonium sulphate precipitation methods

AChR preparation		AChR measured (pmol/ml) DEAE-cellulose filtration	AChR measured (pmol/ml) Ammonium sulphate precipitation
Adult	a	0.33	0.79
	b	0.73	1.07
	c	0.02	1.20
Foetal	a	0.48	0.91
	b	0.02	0.21

AChR was determined in detergent extracts of adult and foetal AChR by the DEAE-cellulose filtration assay and ammonium sulphate precipitation methods, as described in the "Methods" section 7.1 & 7.2.

Figure 14. Determination of percentage saturation of ammonium sulphate required to give maximal precipitation of [^{125}I]α-BGT-AChR complex



saturation of ammonium sulphate, and performing the assay in the usual way. Figure 15 shows that non-specific binding of [^{125}I]α-BGT to the glass fibre discs was linear, and represented 9.4% of the total [^{125}I]α-BGT added initially. The time necessary for maximum formation of [^{125}I]α-BGT - AChR complex prior to the addition of ammonium sulphate was determined by incubating constant amounts of [^{125}I]α-BGT and AChR, adding ammonium sulphate at various times thereafter, followed by incubation for 16 h at 4^{o}C, and filtration on glass fibre discs as previously described (see "Methods" section 7.1). Table 7(a) shows that either [^{125}I]α-BGT - AChR complex formation is very rapid ie. virtually immediate, which did not appear to be the case from the DEAE-cellulose filtration assay (see "Results" section 5.3.1), or that the addition of ammonium sulphate does not stop the reaction immediately, but takes time to reach equilibrium. Table 7(b) shows a variety of combinations of the order of addition of [^{125}I]α-BGT, AChR and ammonium sulphate, and the resulting AChR concentration measured after 16 h at 4^{o}C. This indicates that whatever the order of addition of the components, the resulting measurement of AChR remains the same. Furthermore it is clear that ammonium sulphate cannot be used to stop the reaction between [^{125}I]α-BGT and AChR - this is important for subsequent assays (see "Results" section 5.3).

The optimum time for incubation of [^{125}I]α-BGT - AChR complex with ammonium sulphate to give maximum precipitation of radiolabel was determined, and the results shown in Table 7(c). Incubation for 5 h at 23^{o}C or 4^{o}C gave maximal precipitation of complex (although other results (see Table 7(d)) suggested that 16 h at 4^{o}C was better); further incubation upto 16 h did not affect the result, and

Figure 15. Non-specific precipitation of [^{125}I]α-BGT and retention on GFC filters in the ammonium sulphate assay

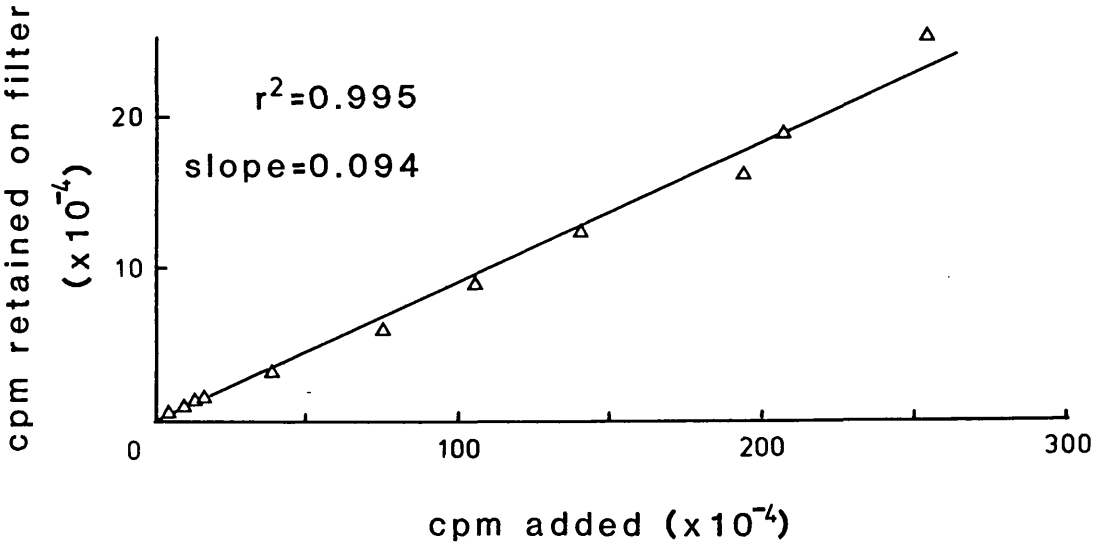


Table 7. (a) Precipitation of [^{125}I]α-BGT - AChR complex by ammonium sulphate

Time of incubation of [^{125}I]α-BGT with AChR before addition of ammonium sulphate (min)	AChR measured (pmol/ml) after incubation with ammonium sulphate for 16 h at 4°C
0	0.56
2	0.75
6	0.66
10	0.63
20	0.65

Table 7. (b) Order of addition of components in ammonium sulphate assay

Initial incubation	Time incubation (min)	Final addition (incubation 16h at 4°C)	AChR measured (pmol/ml)
[^{125}I]α-BGT + AChR	0	$(NH_4)_2\ SO_4$	0.68
[^{125}I]α-BGT + AChR	45	$(NH_4)_2\ SO_4$	0.67
AChR + $(NH_4)_2\ SO_4$	10	[^{125}I]α-BGT	0.65
[^{125}I]α-BGT + $(NH_4)_2\ SO_4$	10	AChR	0.53

Table 7. (c) Time and temperature of incubation of [^{125}I]α-BGT labelled AChR with ammonium sulphate required for maximum precipitation of complex

	AChR measured (pmol/ml)	
	Temperature of incubation	
Incubation time (h)	23°C	4°C
2	1.41	0.64
5	1.46	1.38
16	1.53	1.39

Table 7. (d) Number of glass fibre filter discs for maximum retention of [^{125}I]α-BGT - AChR complex

	AChR measured (pmol/ml)	
Time and temperature	Number of GFC filters	
of incubation	1	2
16 h, 4°C	1.65	1.07
5 h, 23°C	1.30	1.01

incubation for 16 h was often used for convenience.

Increasing the number of GFC filters used did not improve the amount of [^{125}I]α-BGT - AChR complex measured (see Table 7(d)), in fact it led to less complex being retained.

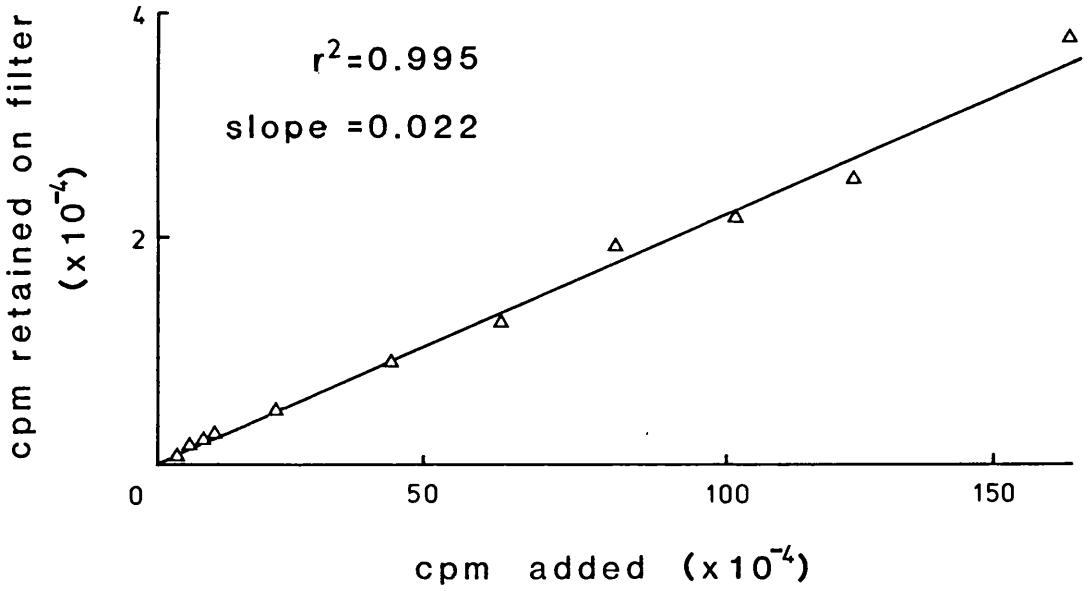
The combination of using 40% ammonium sulphate to precipitate [^{125}I]α-BGT - AChR complex for 16 h at 4^{o}C, and filtering through 1 GFC filter disc, led to a reliable, reproducible method for determination of [^{125}I]α-BGT binding in crude detergent extracts of muscle AChR.

3.2 DEAE-cellulose filtration method

The DEAE-cellulose filtration assay was used mainly for the determination of AChR in purified preparations. Binding of [^{125}I]α-BGT to AChR was shown to be complete after 90 min incubation at 23^{o}C (see "Results" section 5.3.1). Contribution of [^{125}I]α-BGT to the assay blank was assessed by filtering increasing amounts of [^{125}I]α-BGT (0 - 2 pmoles, representing 0 - 20 nM in the assay), in the presence of toxin binding assay buffer (see "Methods" section 7.2) alone. Figure 16 shows that the non-specific binding of [^{125}I]α-BGT to DEAE-cellulose filter discs was linear with increasing amounts of [^{125}I]α-BGT and represented 2.2% of the total [^{125}I]α-BGT added. This value is rather less than the blank value obtained in the ammonium sulphate assay, probably due to some precipitation of [^{125}I]α-BGT protein by ammonium sulphate in addition to non-specific adhesion to glass fibre discs.

There was a suggestion from some experiments (see "Results" section 1.4) that the conditions of the assay were underestimating the amount of [^{125}I]α-BGT binding to DEAE-cellulose filter discs, as

Figure 16. Non-specific binding of [^{125}I]α-BGT to DEAE-cellulose filter discs



compared with gel filtration on Sephadex G-50. The effect of increasing the number of DEAE-cellulose filter discs used, and increasing the time of contact of [^{125}I]α-BGT - AChR with the filters before washing through, both in the assay for measurement of AChR and measurement of biological activity of [^{125}I]α-BGT was therefore investigated. Purified Torpedo AChR was used for these studies, as it provided a more abundant source of AChR than human muscle. Figure 17(a,b) shows that if [^{125}I]α-BGT-AChR is filtered immediately through the DEAE-cellulose filter discs, increasing the number of filter discs used does increase the retention of labelled AChR. If, however, the labelled AChR is allowed to remain in contact with the filter disc for 2 or 5 min before washing through, then the optimum number of discs is 2 and no further [^{125}I]α-BGT can be bound. Therefore, in all further experiments, 2 DEAE-cellulose filter discs were used per test, and the time of contact of labelled AChR with the filter discs was increased to 2 min, instead of filtering immediately.

3.3 Immunoprecipitation method

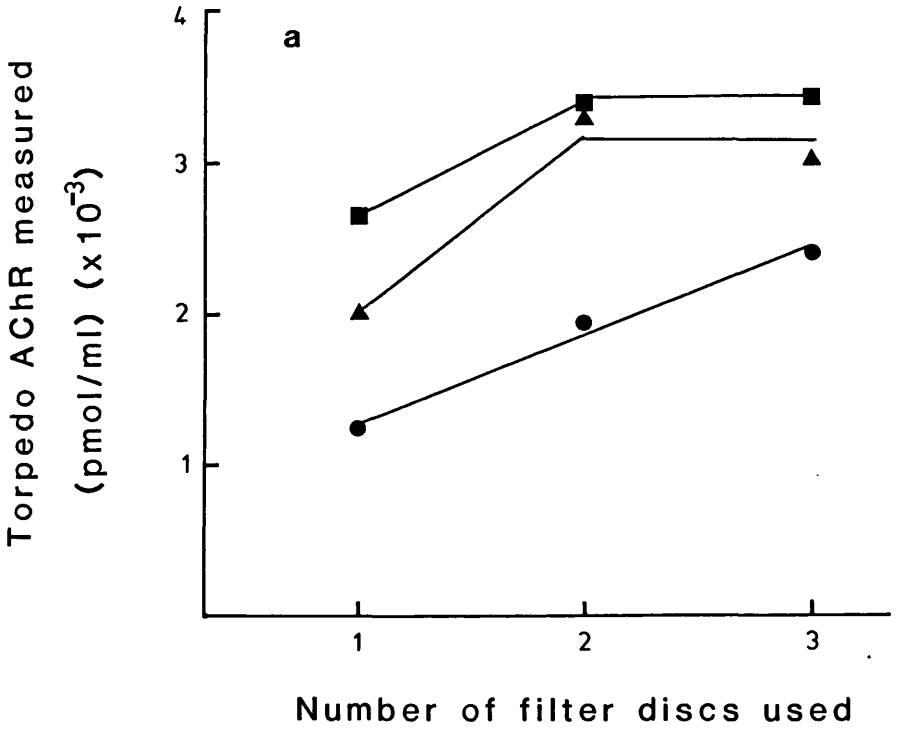
This method was used for determining AChR in both detergent extracts and purified preparations of human adult and foetal AChR. Serum from patient 3 (see "Results" section 4.2) consistently precipitated 95 - 100% of the activity compared with ammonium sulphate or DEAE-cellulose filtration methods (see Table 8).

Figure 17. Optimization of conditions for DEAE-cellulose filtration assay

(a) Determination Torpedo AChR

(b) Determination biological activity [^{125}I]α-BGT

The assays for Torpedo AChR and biological activity of [^{125}I]α-BGT were performed as described in "Methods" sections 7.2 and 1.4 respectively, using appropriate amounts of Torpedo AChR and [^{125}I]α-BGT. After incubation for 90 min at 23^{o}C, samples were filtered through increasing numbers of DEAE-cellulose filter discs after increasing times of contact of the samples with the filters (● 0 min; ▲ 2 min; ■ 5 min) before washing through. Values are means of 2 determinations.


a
Torpedo AChR measured
(pmol/ml) (x10^{-3})
4
3
2
1
1
2
3
Number of filter discs used

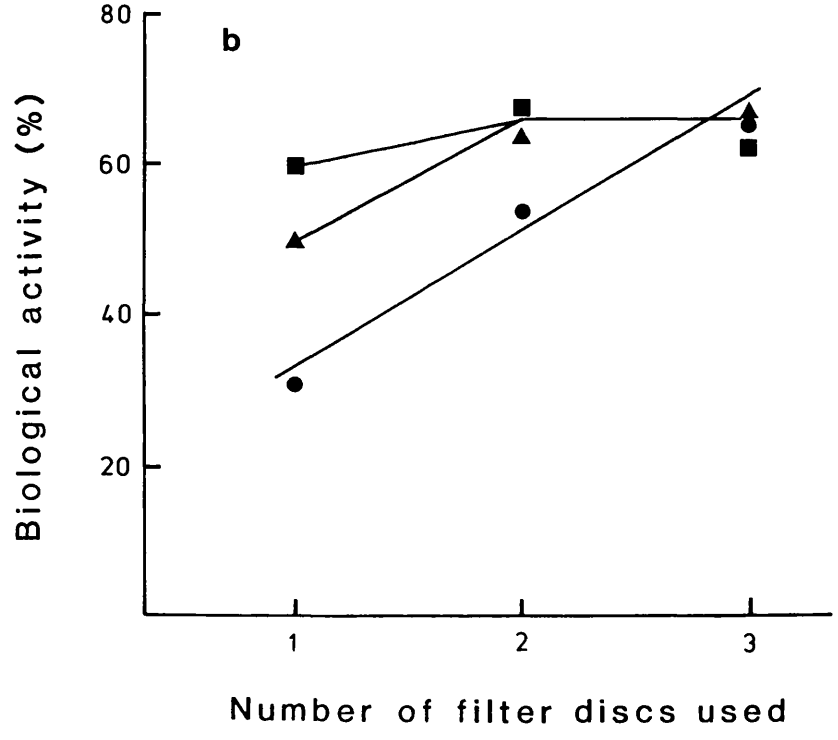

b
Biological activity (%)
80
60
40
20
1
2
3
Number of filter discs used

Table 8. Comparison of ammonium sulphate precipitation and DEAE-cellulose filtration assays with the immunoprecipitation method

AChR used	Result in immunoprecipitation assay as a percentage of result obtained from:- Detergent extracts in ammonium sulphate precipitation assay	Purified preparations in DEAE-cellulose filtration assay
Adult	90 ± 4.0	104 ± 3.3
Foetal	95 ± 5.5	109 ± 4.3

Values given represent mean ± SE.

4. Specific features of antibody patterns in myasthenic patients.

4.1 Radioimmunoassay of anti-(AChR) antibodies.

The radioimmunoassay for the determination of anti-(AChR) antibodies in the sera of myasthenic patients involves specific labelling of human adult skeletal muscle AChR with radiolabelled snake venom α-toxin, followed by the use of an excess of the labelled AChR to bind all anti-(AChR) antibodies present in the patient's serum, and precipitation of the complex so formed with a second antibody (see Figure 6, "Introduction" p. 30). Assay of normal volunteers gave values of 0.024 ± 0.007 nM α-BGT binding sites ($\pm$SE, duplicate assays on 20 individuals), while anti-(AChR) antibody titres in clinically diagnosed myasthenic patients ranged from 0 - 600 nM α-BGT binding sites, with a mean value of 22 nM sites. Approximately 90% of myasthenic patients show elevated serum levels of anti-(AChR) antibodies (Lindstrom et al., 1976a), the assay therefore provides a useful aid in the diagnosis of the disease.

The assay is composed of several stages, and many aspects of published procedures differ widely between authors. I became involved in the development and evaluation of the final stages of a reproducible RIA procedure which is suitable for routine application. As this work was done in close collaboration with other workers, it is not detailed separately in the present thesis, but the results can be seen in the bound paper "An assessment of RIA procedures for determination of anti-(AChR) antibodies in the sera of patients with myasthenia gravis."

The main part of the present section uses this RIA to investigate specific features of antibody patterns in myasthenic patients. For some experiments it was necessary to purify IgG from

sera by ammonium sulphate precipitation and ion exchange chromatography (see "Methods" section 5). Anti-(AChR) antibodies were determined in myasthenic sera before purification and in the IgG fraction after purification (see "Methods" section 8). The recoveries of specific antibody activity and yields of IgG from a series of preparations are given in Table 9.

The second antibody for precipitating immune complexes in the RIA was prepared by immunizing goats wih human IgG purified from pooled normal human serum (see "Methods" section 6). The resulting serum was monitored for the production of antibodies by the RIA procedure given (see "Methods" section 8). Figure 18 shows a typical saturation curve for the precipitation of a fixed concentration of AChR - anti-(AChR) antibody complex by increasing volumes of goat anti-(human IgG) antiserum. Repeated injections of IgG were given until the volume of antiserum required to precipitate 5 µl of myasthenic serum was less than 100 µl. Table 10 shows a summary of the results obtained from each series of immunizations.

4.2 Patient-specific antibody patterns.

14 myasthenic patients representing differing disease states were studied for varying times, from 1 - 60 months. All patients underwent a series of plasma exchange and immunosuppression therapy during the period of investigation, and this provided the opportunity to monitor variations in antibody titres accompanying major changes in clinical state.

There was considerable variation in the anti-(AChR) antibody titres between patients, whether human AChR or rat AChR was used as antigen in the RIA. Neither the antibody titre measured against

Table 9. Recoveries of specific anti-(AChR) antibody and yield of IgG from myasthenic sera

Patient number	Anti-(AChR) antibody (pmoles [^{125}I]α-BGT binding sites) in serum	IgG	Recovery specific antibody (%)	yield IgG (mg)
6	182	57	31	56
10	510	411	82	80
11	76	40	53	10
14	54	3	6	22

Figure 18. Saturation curve for precipitation of receptor-antibody complex by increasing volumes of anti-(human IgG) antiserum

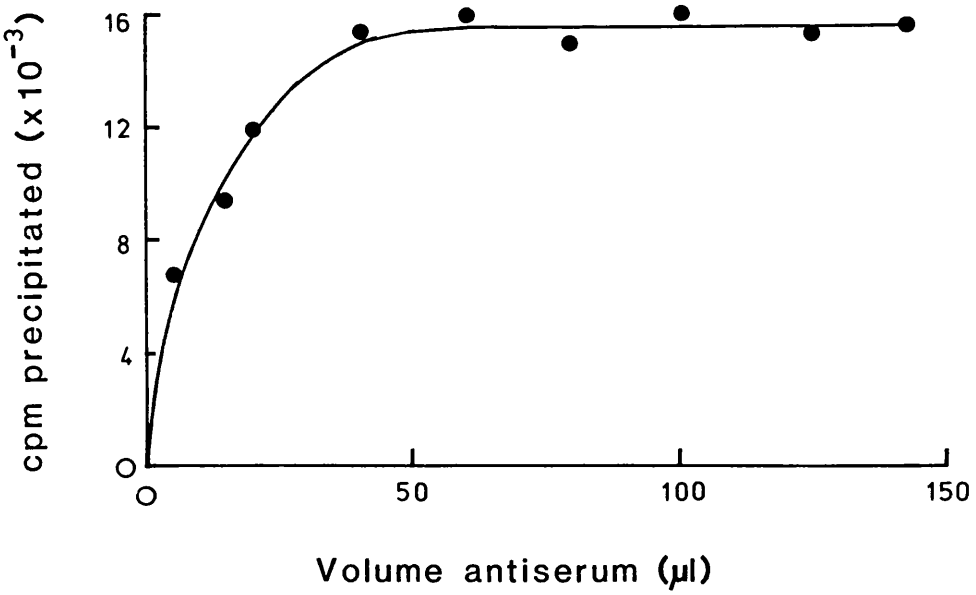


Table 10. Summary of results obtained from preparation of goat anti-(human IgG) antiserum

Goat	Number of injections	Volume antiserum required for precipitation of AChR-Ab complex (µl)	Volume antiserum obtained
Stanley	12	80	1.1L
Blossom	4	60	1.0L
Cyril	4	30	1.3L

human AChR nor that against rat AChR correlated with the severity of the disease (see Figure 19). However, it was found that the antibody titre measured against human AChR was always greater than if extra-junctional rat AChR (EJ-rat AChR) was used, which was, in turn, always higher than with junctional rat AChR (J-rat AChR) (see Figure 20). The anti-(EJ-rat AChR) antibody titre was 0 - 63% of the anti-(human AChR) antibody titre (see Figure 21); whereas the anti-(J-rat AChR) antibody titre was 0 - 100% of the anti-(EJ-rat AChR) antibody titre (see Figure 22). Although the antibody titres for an individual patient were found to vary considerably with time, the ratio of titres, both anti-(EJ-rat AChR) : anti-(human AChR) and anti-(J-rat AChR) : anti-(EJ-rat AChR) were relatively constant for an individual patient over the period of study, despite varying treatment regimes. This is illustrated in Figure 23 (a - n).

If the RIA was modified by adding excess myasthenic sera to precipitate a limited amount of [^{125}I]α-BGT - labelled human AChR, it was found that the amount precipitated varied between patients (15 - 100%), but was constant for a given patient over the period of study. Serum from one particular patient (patient 3) constantly precipitated 100% of the available labelled AChR, as judged by DEAE-cellulose and ammonium sulphate precipitation assays (see "Results" section 3), so others were expressed as a percentage of this (see Figure 24). The relative abilities of myasthenic sera to precipitate [^{125}I]α-BGT - labelled human AChR provides further evidence for the existance of a patient-specific antibody pattern. Possible reasons for the percentage precipitation effect are discussed in the next section. A summary of these data is given in Table 11.

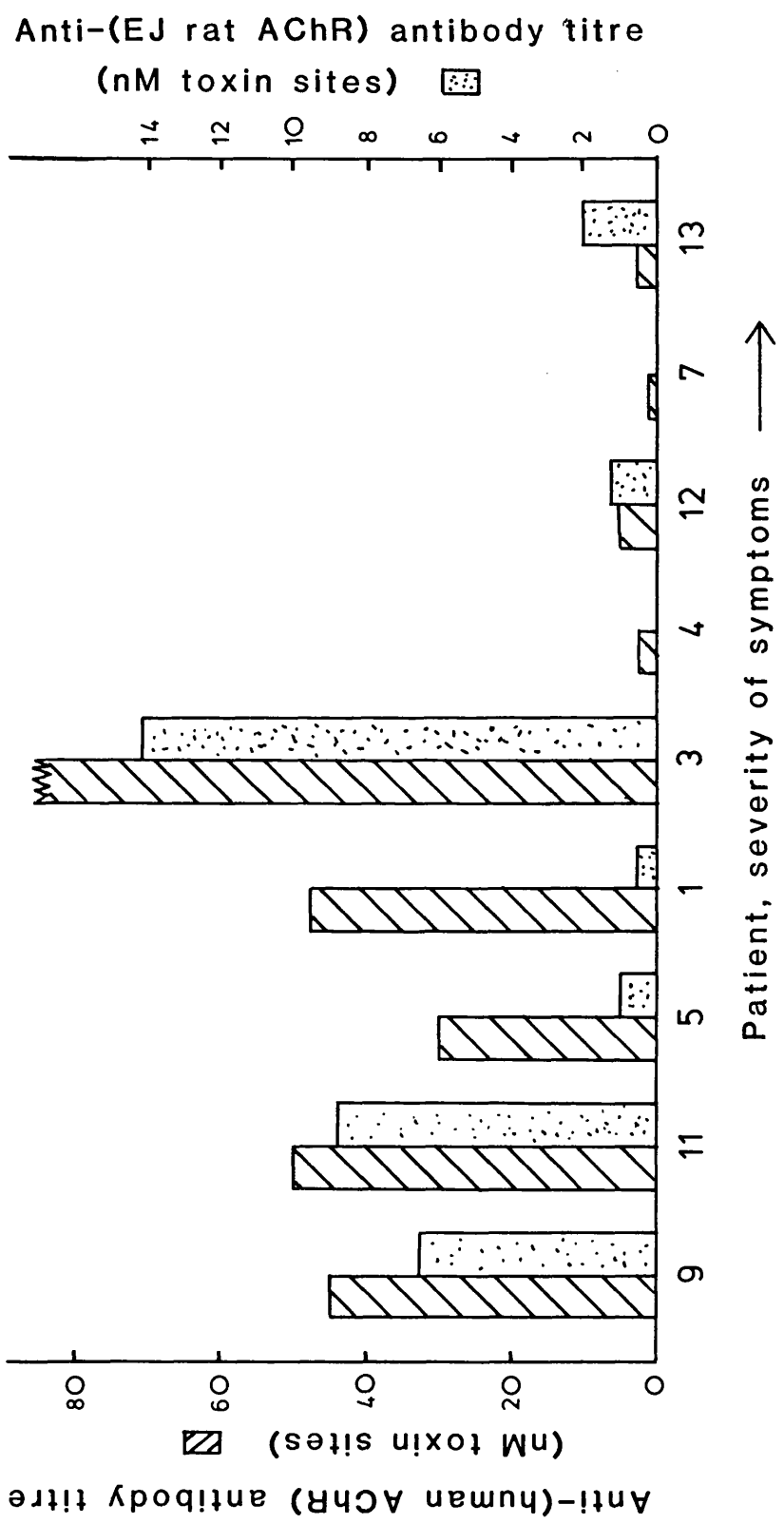


Figure 19. Anti-(human AChR) and anti-(extra-junctional rat AChR) antibody titres from 9 myasthenic patients against disease severity

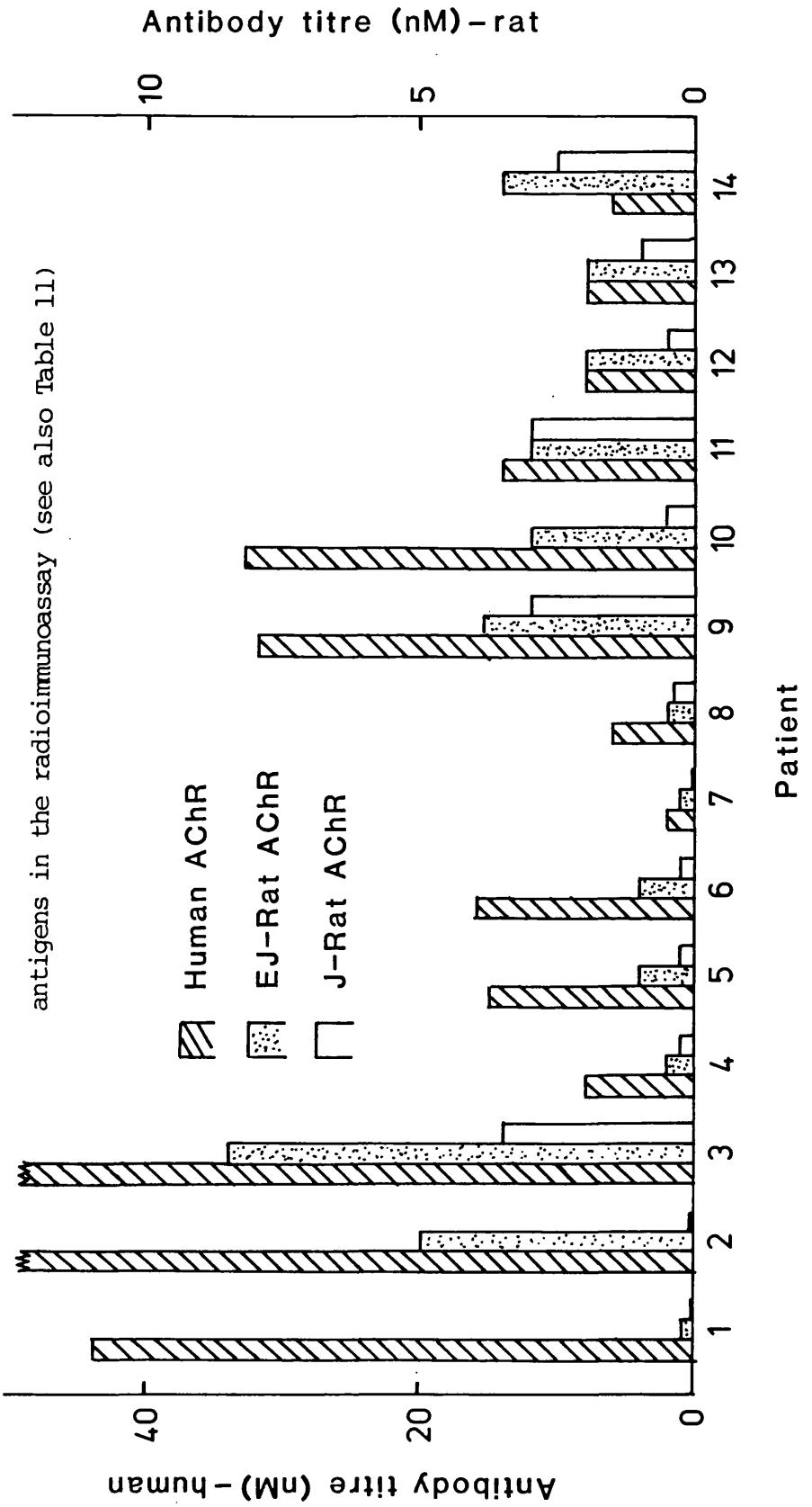


Figure 20. Comparison of anti-(AChR) antibody titres obtained using human, extrajunctional rat and junctional rat AChRs as antigens in the radioimmunoassay (see also Table 11)

Figure 21. Ratios of anti-(extrajunctional rat AChR) : anti-(human AChR) antibody titres for repeat serum samples from 14 different myasthenic patients.

The heights of the columns represent mean ratios, the error bars surmounting the columns represent ±SE, and the number of samples assayed is shown above each column (see also Table 11).

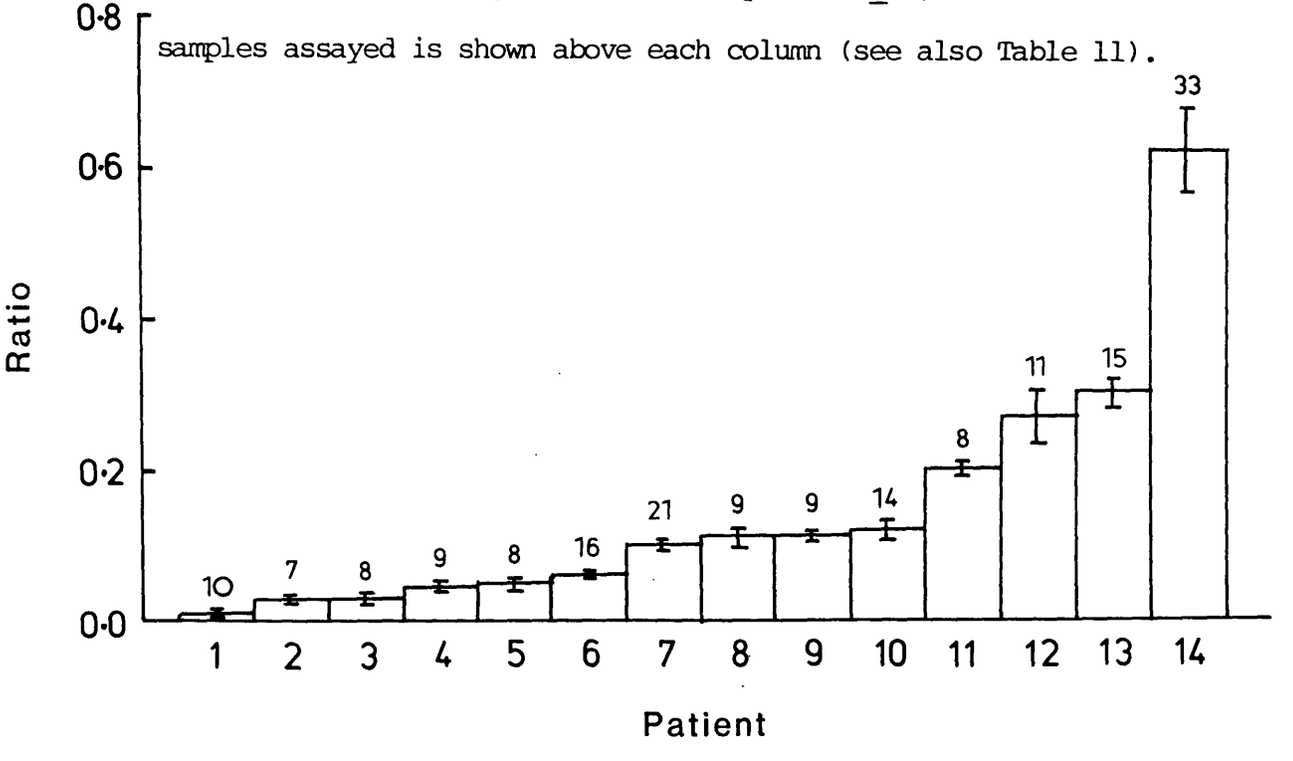


Figure 22. Ratios of anti-(junctional rat AChR) : anti-(extrajunctional rat AChR) antibody titres for repeat serum samples from 14 different myasthenic patients.

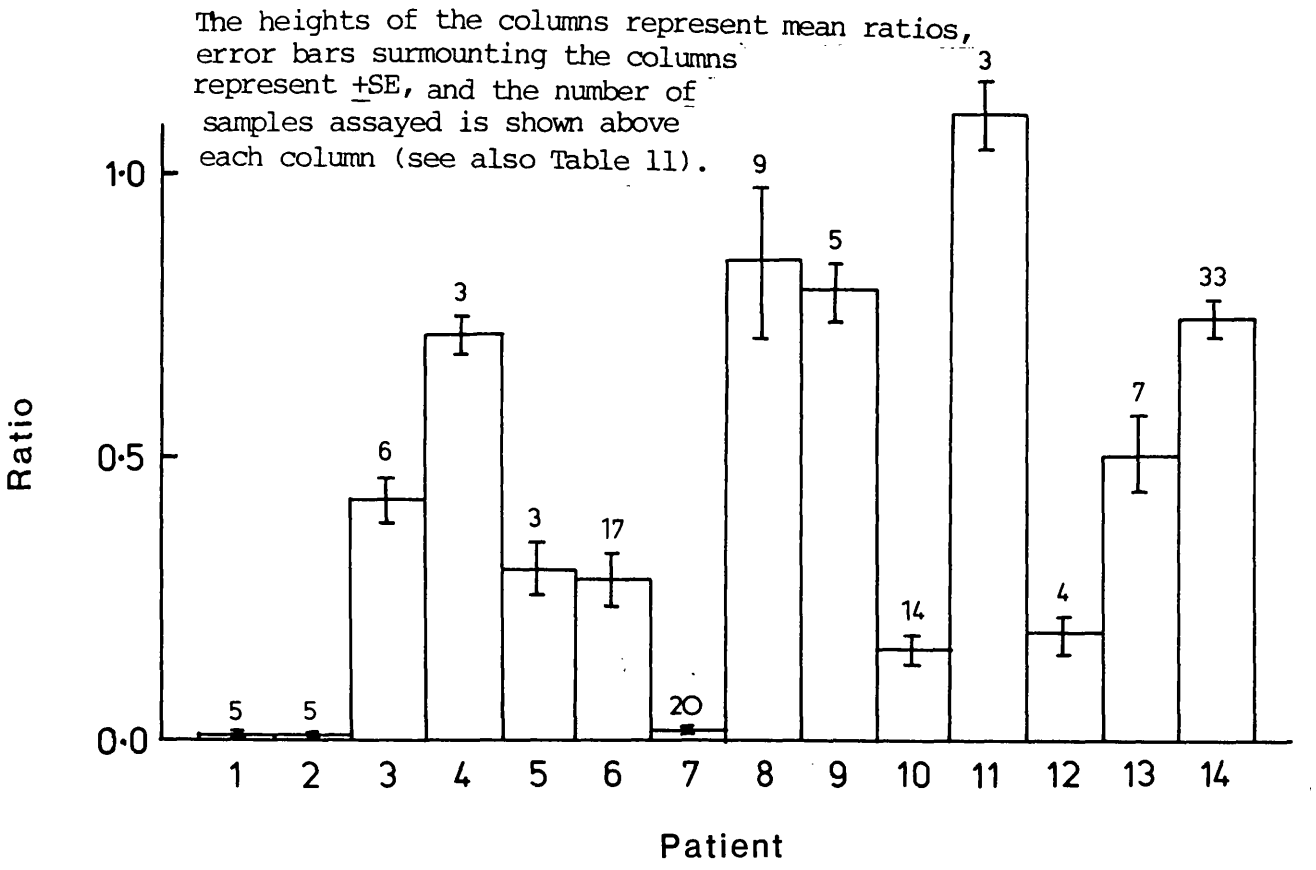


Figure 23. Anti-(human AChR) antibody titres and ratios of anti-(extrajunctional rat AChR) : anti-(human AChR) antibody titres for 14 myasthenic patients over various time periods:-

(a) Patient 1 - 33 months

(b) Patient 2 - 35 months

(c) Patient 3 - 26 months

● Pre-plasmapheresis
▲ Post-plasmapheresis
} Anti-(human AChR) antibody titre

○ Pre-plasmapheresis
△ Post-plasmapheresis
} Ratio anti-(EJ rat AChR) : anti-(human AChR) antibody titres

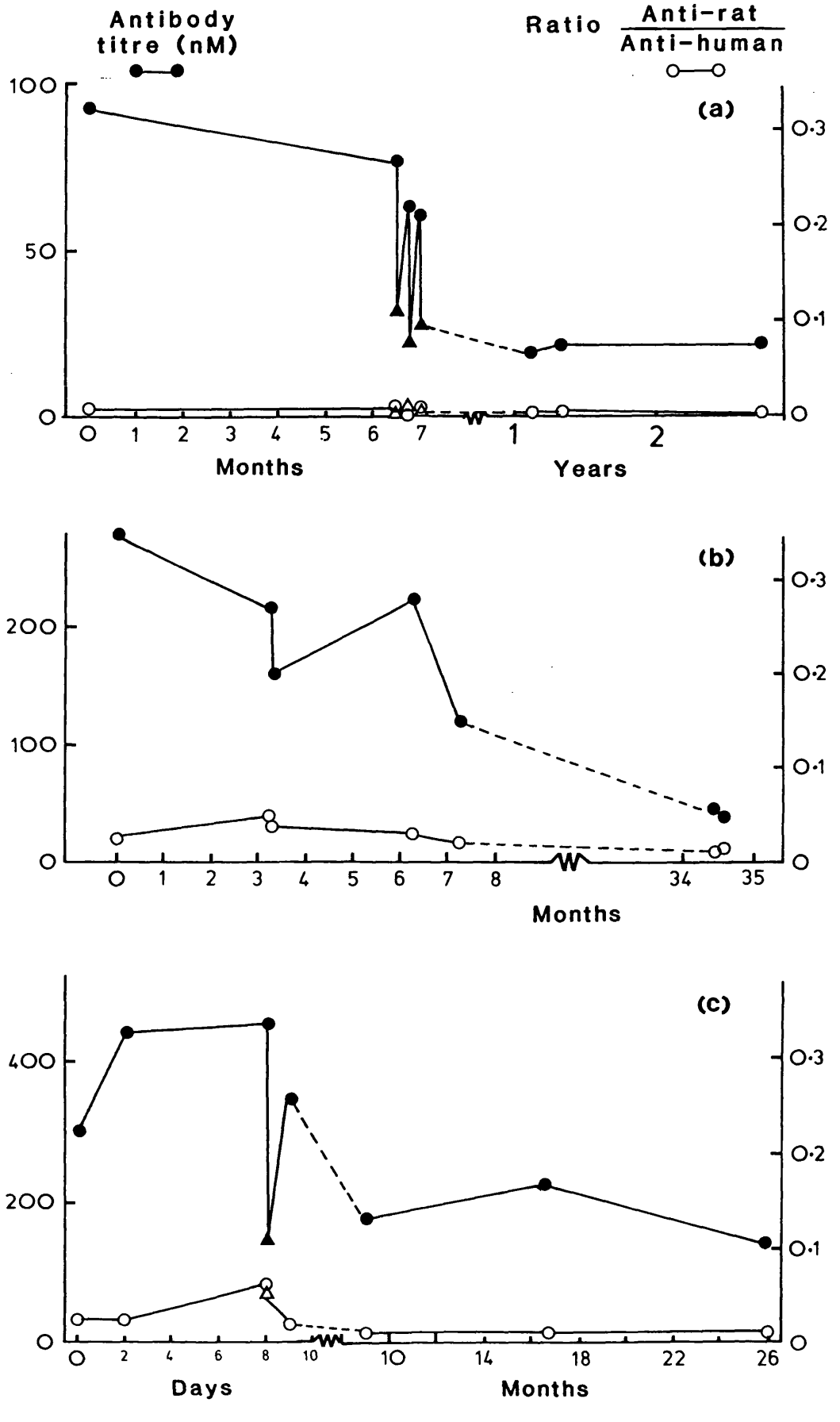

Antibody titre (nM)
Ratio Anti-rat / Anti-human
(a)
100
50
0
0·3
0·2
0·1
0
0 1 2 3 4 5 6 7 1 2
Months
Years
(b)
200
100
0
0·3
0·2
0·1
0
0 1 2 3 4 5 6 7 8 34 35
Months
(c)
400
200
0
0·3
0·2
0·1
0
0 2 4 6 8 10 10 14 18 22 26
Days
Months

Figure 23. Anti-(human AChR) antibody titres and ratios of anti-(extrajunctional rat AChR) : anti-(human AChR) antibody titres for 14 myasthenic patients over various time periods:-

(d) Patient 4 - 17 months

(e) Patient 5 - 2 months

(f) Patient 6 - 4 years

● Pre-plasmapheresis
▲ Post-plasmapheresis
} Anti-(human AChR) antibody titre

○ Pre-plasmapheresis
△ Post-plasmapheresis
} Ratio anti-(EJ rat AChR) : anti-(human AChR) antibody titres

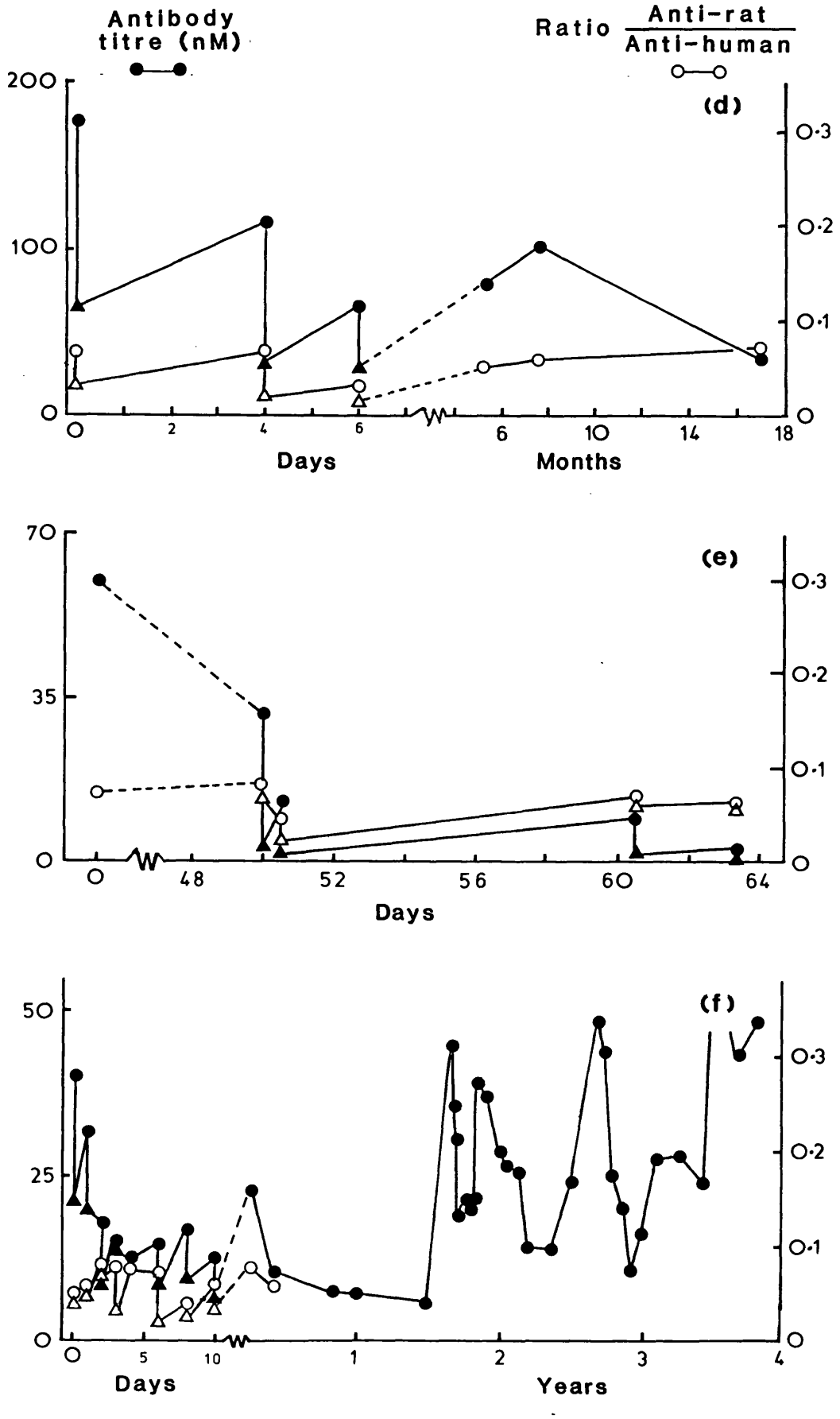

Antibody titre (nM)
Ratio Anti-rat / Anti-human
(d)
200
100
0
0·3
0·2
0·1
0
2
4
6
6
10
14
18
Days
Months
(e)
70
35
0
48
52
56
60
64
Days
(f)
50
25
0
5
10
1
2
3
4
Days
Years

Figure 23. Anti-(human AChR) antibody titres and ratios of anti-(extrajunctional rat AChR) : anti-(human AChR) antibody titres for 14 myasthenic patients over various time periods:-

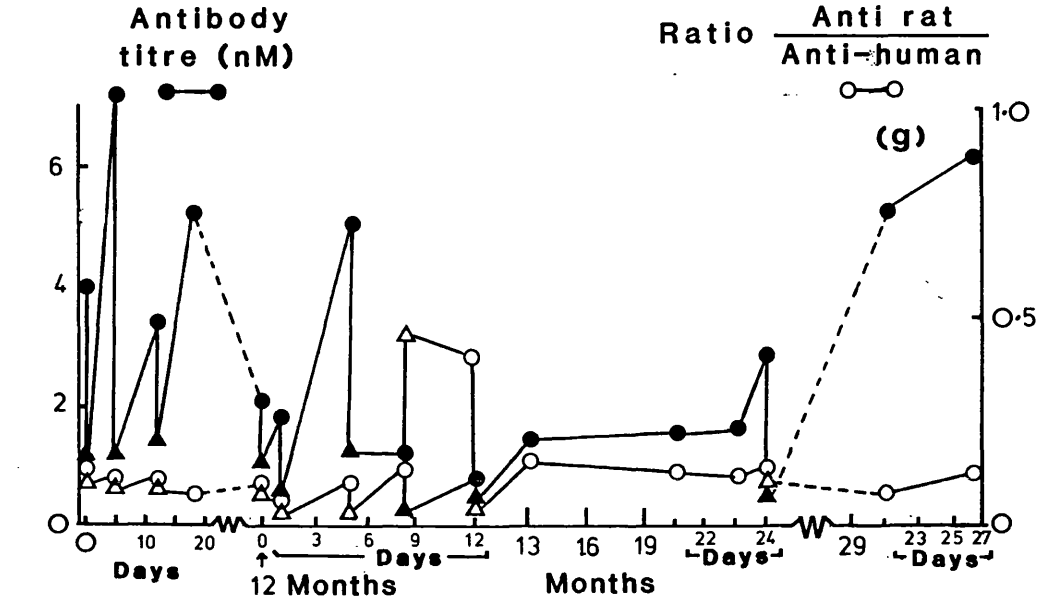
(g) Patient 7 - 30 months

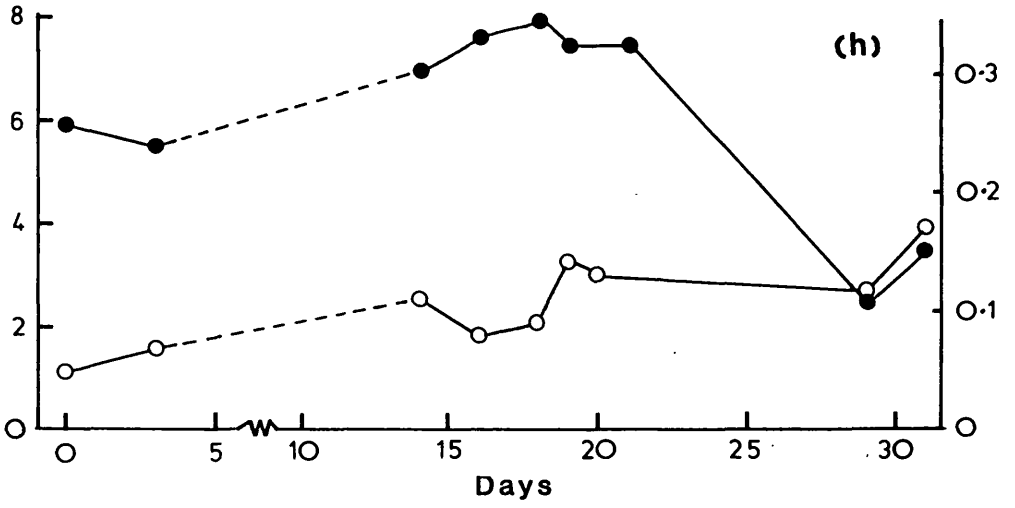
(h) Patient 8 - 31 days

(i) Patient 9 - 25 months

● Pre-plasmapheresis
▲ Post-plasmapheresis
} Anti-(human AChR) antibody titre

o Pre-plasmapheresis
Δ Post-plasmapheresis
} Ratio anti-(EJ rat AChR) : anti-(human AChR) antibody titres


Antibody titre (nM)
Ratio Anti rat / Anti-human
(g)
Days
12 Months
Months
Days
Days
Days


(h)
Days

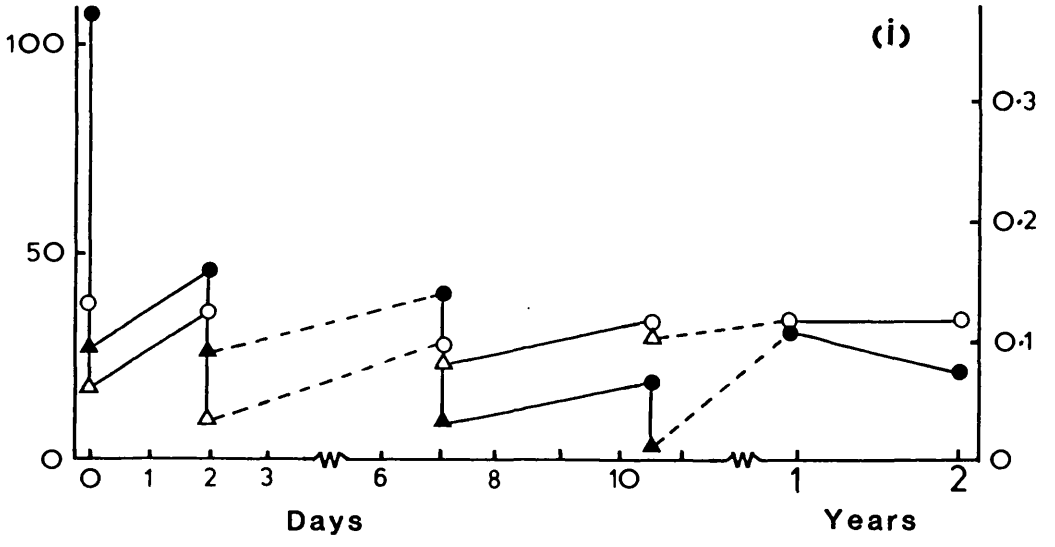

(i)
Days
Years

Figure 23. Anti-(human AChR) antibody titres and ratios of anti-(extrajunctional rat AChR) : anti-(human AChR) antibody titres for 14 myasthenic patients over various time periods:-

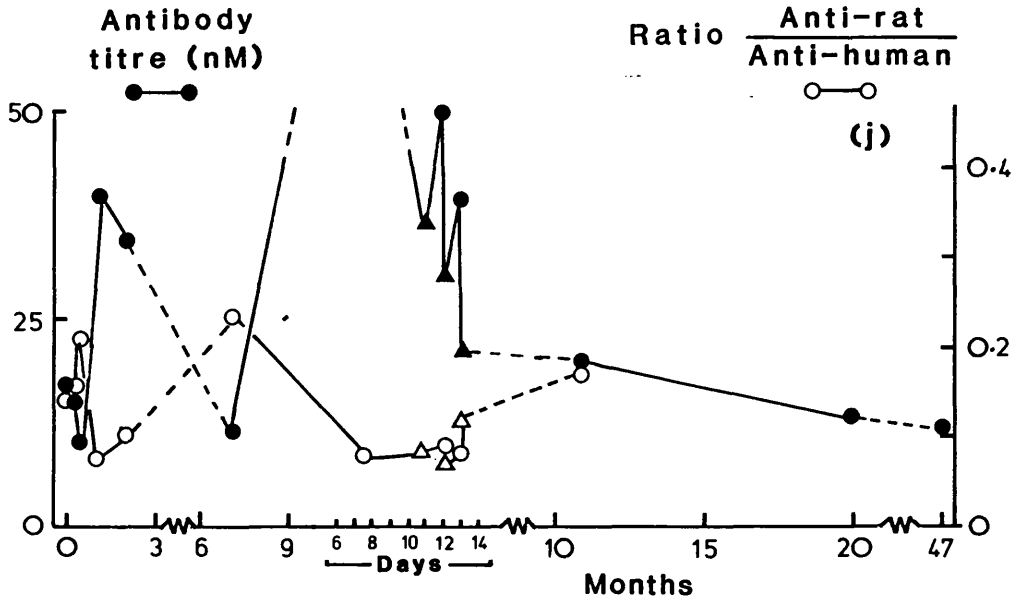
(j) Patient 10 - 20 months

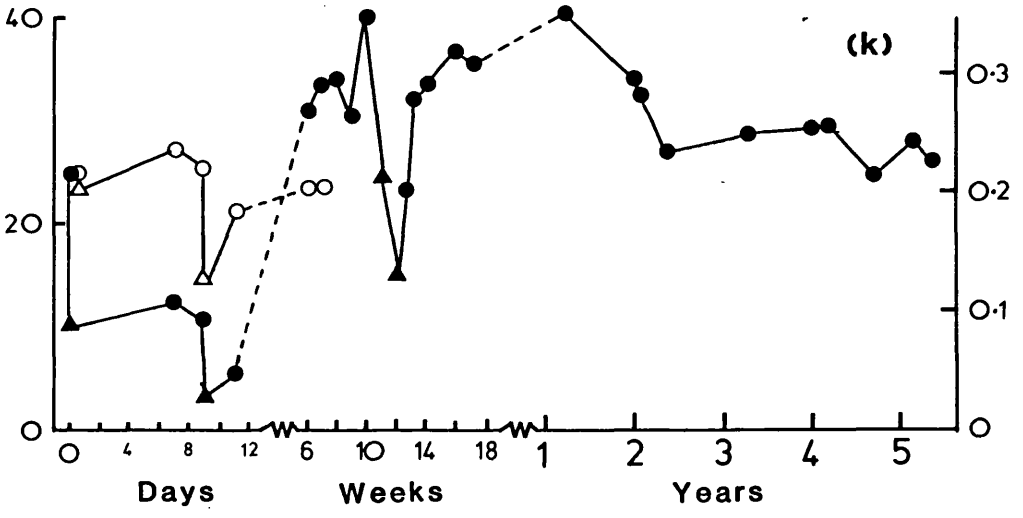
(k) Patient 11 - 5 years

(l) Patient 12 - 16 months

● Pre-plasmapheresis
▲ Post-plasmapheresis
} Anti-(human AChR) antibody titre

○ Pre-plasmapheresis
△ Post-plasmapheresis
} Ratio anti-(EJ rat AChR) : anti-(human AChR) antibody titres


Antibody titre (nM)
Ratio Anti-rat / Anti-human
(j)
50
25
0
0·4
0·2
0
0 3 6 9 6 8 10 12 14 10 15 20 47
Days
Months


(k)
40
20
0
0·3
0·2
0·1
0
0 4 8 12 6 10 14 18 1 2 3 4 5
Days
Weeks
Years

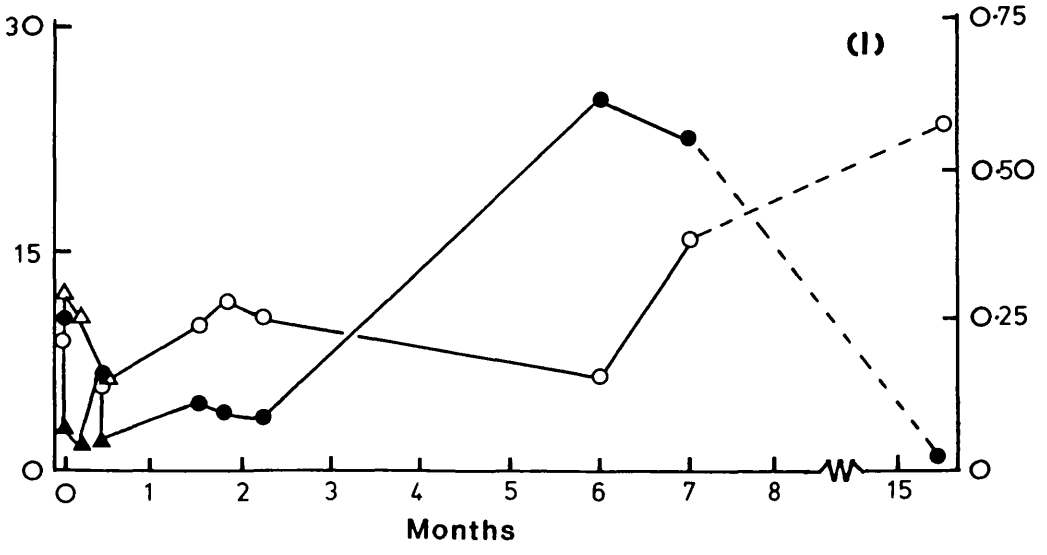

(l)
30
15
0
0·75
0·50
0·25
0
0 1 2 3 4 5 6 7 8 15
Months

Figure 23. Anti-(human AChR) antibody titres and ratios of anti-(extrajunctional rat AChR) : anti-(human AChR) antibody titres for 14 myasthenic patients over various time periods:-

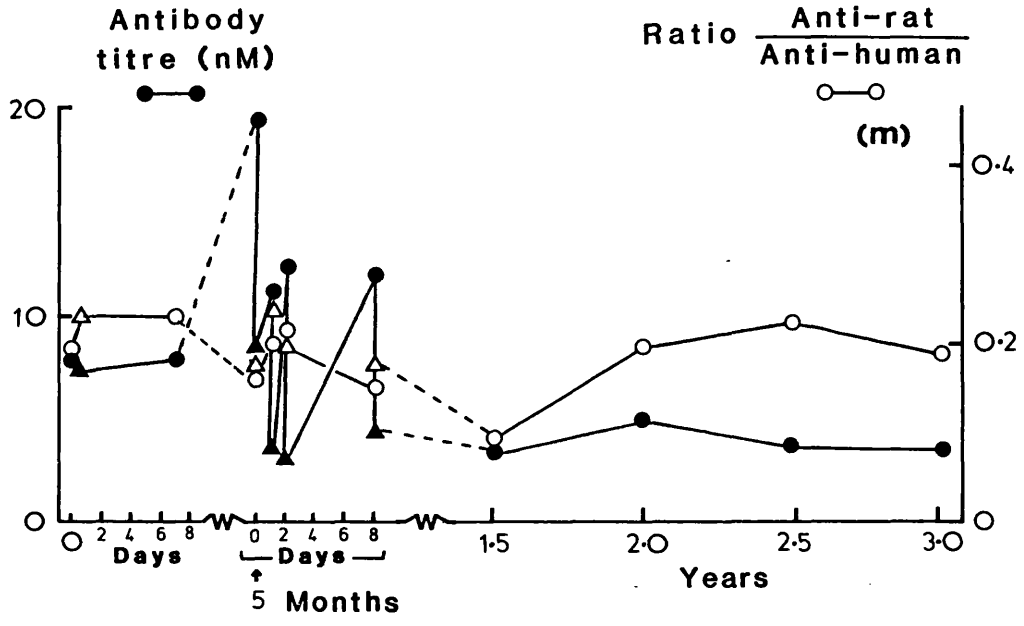
(m) Patient 13 - 3 years

(n) Patient 14 - 1 year

● Pre-plasmapheresis
▲ Post-plasmapheresis
} Anti-(human AChR) antibody titre

○ Pre-plasmapheresis
△ Post-plasmapheresis
} Ratio anti-(EJ rat AChR) : anti-(human AChR) antibody titres

Results from all these graphs are summarized in Table 11.


Antibody titre (nM)
Ratio Anti-rat / Anti-human
(m)
20
10
0
0·4
0·2
0
2 4 6 8
Days
0 2 4 6 8
Days
5 Months
1·5
2·0
2·5
3·0
Years

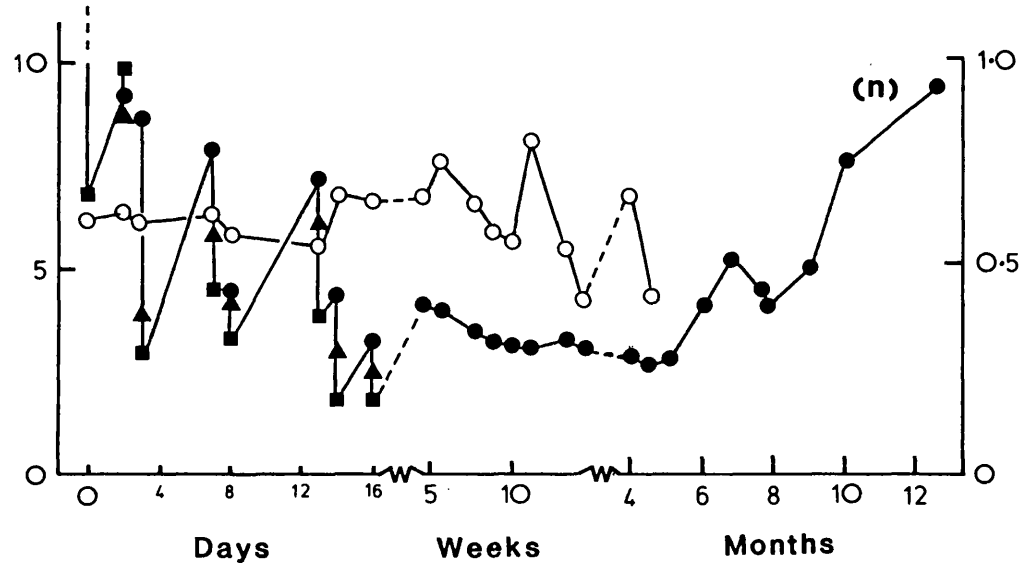

(n)
10
5
0
1·0
0·5
0
4
8
12
16
5
10
4
6
8
10
12
Days
Weeks
Months

Figure 24. Precipitation of [^{125}I]α-BGT labelled human AChR by repeat serum samples from 14 different myasthenic patients.

Excess of the serum was used, with goat anti-(human IgG) antiserum, to precipitate labelled AChR. The heights of the columns represent mean percentages of the amount of labelled AChR precipitated by serum from patient 3. The error bars surmounting the columns represent $\pm$SE, and the number of samples assayed is shown above each column (see also Table 11).

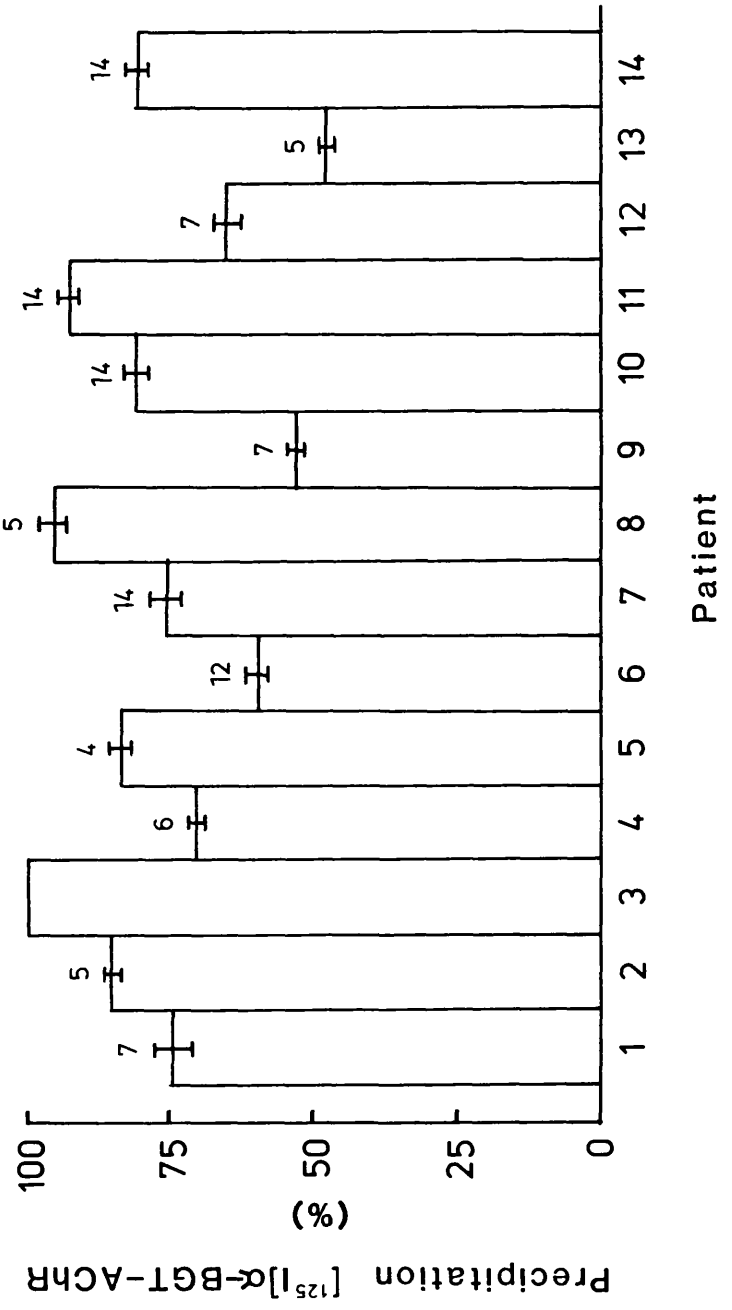

Precipitation [125I]α-BGT-AChR
(%)
100
75
50
25
0
Patient
1 2 3 4 5 6 7 8 9 10 11 12 13 14
7 5 6 4 12 14 5 7 14 14 7 5 14

Table 11. Summary of anti-(AChR) antibody titres and ratios of anti-(AChR) antibody titres obtained using human adult AChR, extra-junctional rat AChR, or junctional rat AChR as antigen in the RIA of myasthenic sera; and percentage precipitation of human AChR by excess myasthenic sera.

H = anti-(human adult AChR) antibody titre

JR = anti-(junctional rat AChR) antibody titre

EJR = anti-(extra-junctional rat AChR) antibody titre

Antibody titres are expressed as nM α-BGT binding sites

* A large proportion of assays done on samples from these patients were by T.S.M.

		1 *	2
	Range H	19 - 93	49 - 279
	Mean H	44	157
	Range EJR	0 - 1	0.5 - 9
	Mean EJR	0.3	4.9
	Range JR	0	0
	Mean JR	0	0
Ratio EJ:H	mean	0.007	0.027
	SEM	0.004	0.006
	no. samples	10	7
Ratio J:EJ	mean	0.000	0.000
	SEM	0.000	0.000
	no. samples	5	5
Human AChR precipitated	mean	75	86
(%)	SEM	3.2	0.7
	no. samples	7	5
"Inhibition factor"		-	-
Period of study (months)		33	35

Patient				
3	4 *	5 *	6	7
137 - 451	3 - 18	1 - 60	6 - 60	0.2 - 6
277	8	15	23	2
1 - 28	0.04 - 1.2	0 - 4.4	0.2 - 2	0 - 0.7
8.4	0.4	1	1	0.2
1 - 10	0.2 - 0.8	0 - 1.2	0 - 1	0 - 0.1
3.5	0.3	0.3	0.3	0.0
0.028	0.046	0.053	0.059	0.099
0.008	0.008	0.010	0.005	0.008
8	9	8	16	21
0.422	0.710	0.300	0.280	0.015
0.040	0.042	0.050	0.049	0.011
6	3	3	17	20
100	71	85	60	76
-	1.1	2.1	1.9	3.4
-	6	4	12	14
-	-	-	+	-
26	17	2	59	30

		8	9 *
	Range H	2 - 8	2 - 109
	Mean H	6	32
	Range EJR	0.3 - 1.1	0.3 - 15
	Mean EJR	0.6	3.7
	Range JR	0.1 - 0.8	0.2 - 1.2
	Mean JR	0.5	2.9
	Ratio EJ:H mean	0.110	0.116
	SEM	0.013	0.007
	no. samples	9	10
	Ratio J:EJ mean	0.837	0.788
	SEM	0.137	0.056
	no. samples	9	5
Human AChR precipitated (%)	mean	96	53
	SEM	2.1	1.6
	no. samples	5	7
	"Inhibition factor"	-	+
	Period of study (months)	1	25

Patient				
10	11	12 *	13 *	14
10 - 85	3 - 40	1 - 25	3 - 20	2 - 31
33	26	8	8	12
2.2 - 4	0.4 - 7.4	0.3 - 8.8	0.5 - 5.0	1 - 16
3.2	3.0	2.0	2.2	3.7
0 - 1	0.4 - 7.4	0.1 - 2.0	0.3 - 2	1 - 13
0.5	3.3	0.4	1.1	2.4
0.122	0.202	0.279	0.297	0.617
0.014	0.012	0.037	0.016	0.020
14	8	11	15	33
0.162	1.100	0.188	0.502	0.742
0.025	0.070	0.034	0.069	0.024
14	3	4	7	33
82	93	67	48	82
1.9	1.9	2.3	0.6	2.1
14	14	7	5	14
-	-	-	+	+
36	61	16	36	12

4.3 Percentage precipitation of [^{125}I]α-BGT - labelled AChR by excess myasthenic serum.

Sera from myasthenic patients, when used in excess, vary in their ability to precipitate all available [^{125}I]α-BGT - labelled AChR complex. Values vary from 15 - 100% (see "Results" section 4.2, Figure 24), with a mean value of 75 $\pm$ 2.3 ($\pm$SE, 73 determinations). Initially, addition of the various components at the different stages of the RIA procedure was checked to ensure that incomplete precipitation of [^{125}I]α-BGT - labelled AChR was not a result of the use of sub-optimal conditions in the assay. The AChR is known to be fully labelled with [^{125}I]α-BGT after the time and temperature of incubation used and with the concentration of [^{125}I]α-BGT used (see Figure 42), so the effect cannot be explained by incomplete labelling of the receptor. The percentage precipitation does not appear to correlate with the antibody titre; this is shown in Figure 25. IgG purified from 4 myasthenic sera were no more effective than the original serum in precipitating the available [^{125}I]α-BGT - labelled AChR (see Table 12). The highest titre serum represents a 1000-fold molar excess of antibodies over [^{125}I]α-BGT-AChR, so incomplete precipitation is unlikely to result from insufficient antibody. This was confirmed by increasing the volume of serum or IgG used to precipitate the [^{125}I]α-BGT labelled AChR complex, which did not increase the percentage precipitation achieved. Increasing the amount of goat anti-(human IgG) antiserum added to precipitate the labelled receptor-antibody complex also had no effect on the percentage precipitation (see Figure 26(a,b)), so the results do not arise from abnormally high concentrations of IgG being present.

Figure 25. Relationship between percentage precipitation of [^{125}I]α-BGT labelled AChR by excess myasthenic serum and anti-(AChR) antibody titre

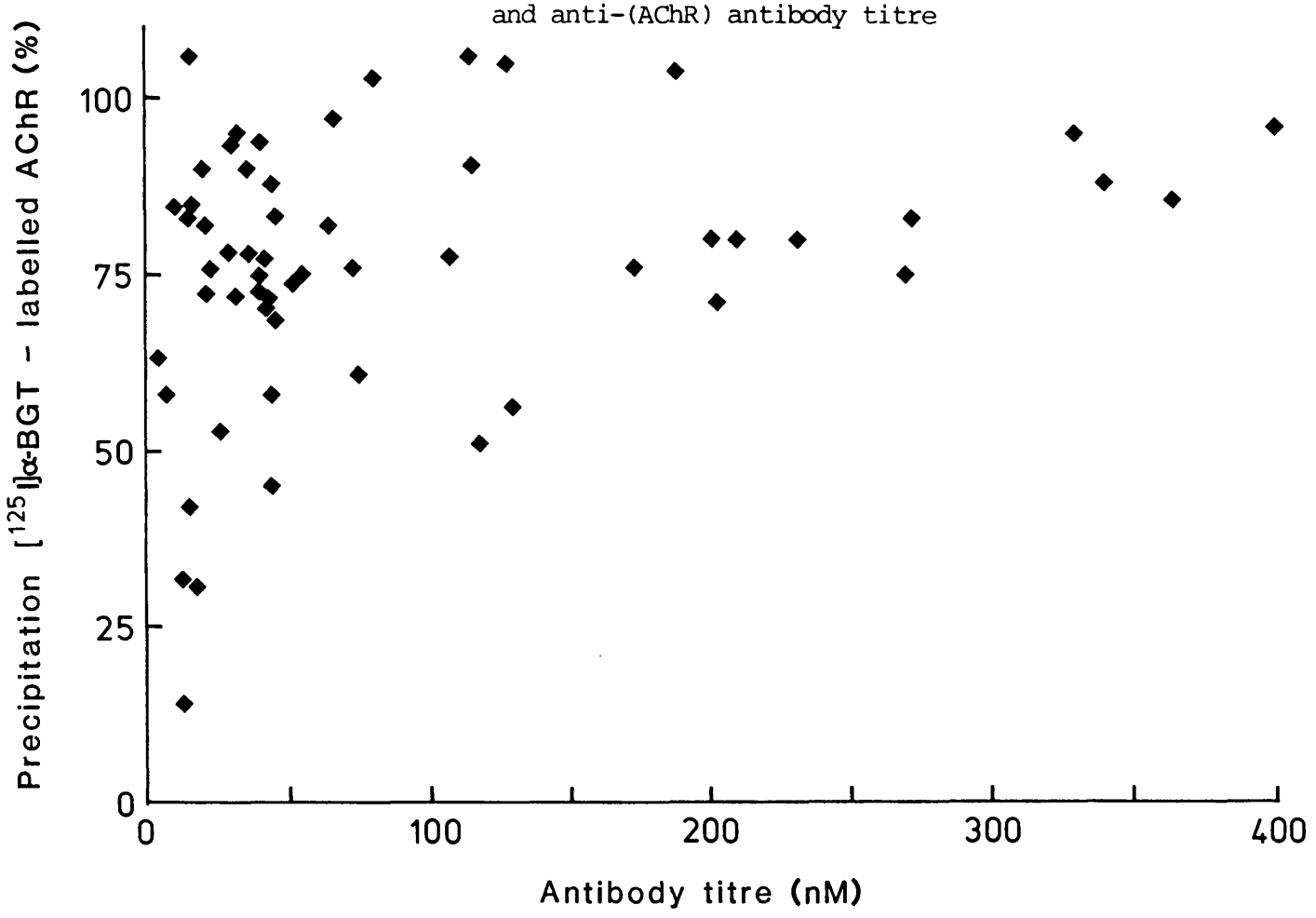


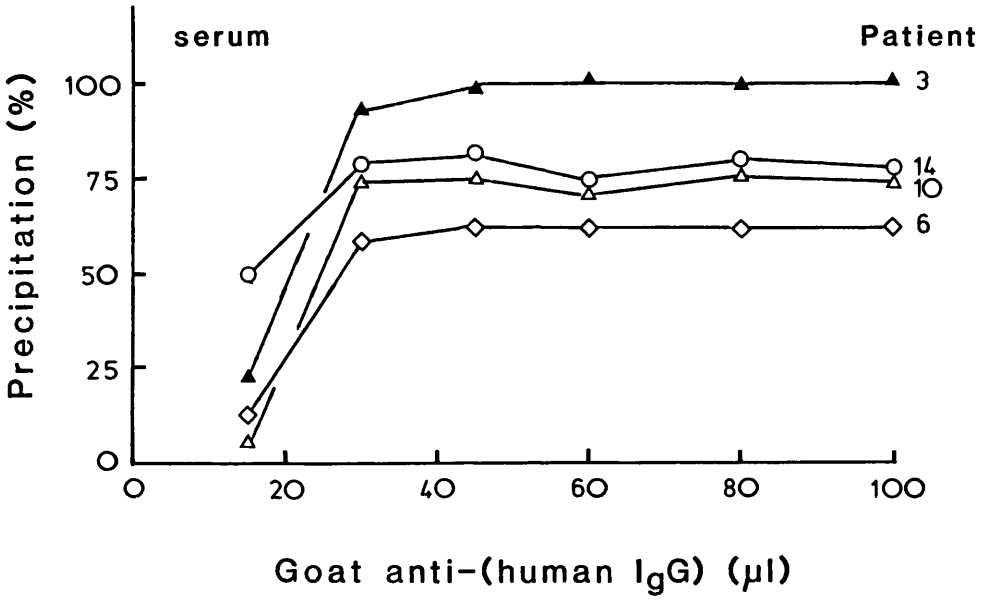
Table 12. Percentage precipitation of [^{125}I]α-BGT - labelled AChR by myasthenic serum and IgG

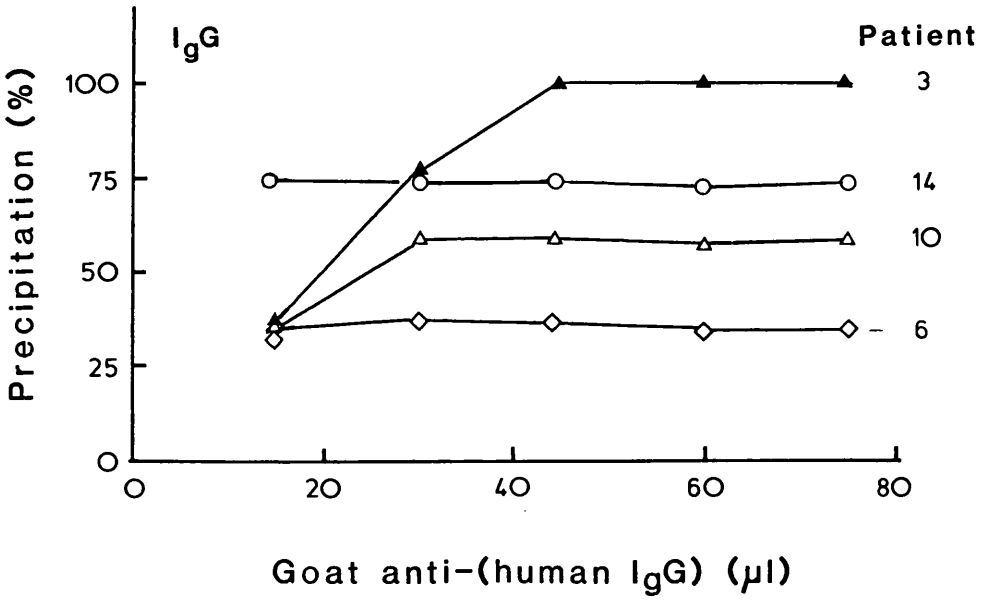
	Percentage precipitation of [^{125}I]α-BGT - AChR			
	serum		IgG	
Patient	mean	SE	mean	SE
13	70	2.0	62	2.0
6	59	3.5	62	3.0
14	78	1.5	83	2.0
10	84	2.0	86	1.5

Each value is the mean of 4 determinations.

Figure 26. Effect of increasing the amount of goat anti-(human IgG) on the percentage precipitation of [^{125}I]α-BGT labelled AChR by myasthenic serum or IgG

Excess serum or IgG (5 µl) from each of 4 MG patients was used to precipitate a constant amount of [^{125}I]α-BGT labelled human adult AChR (0.02 pmol, 100 µl), in the presence and absence of benzoquinonium chloride, as described in "Methods" section 8. Increasing volumes of goat anti-(human IgG) antiserum were added to effect precipitation of the labelled AChR-antibody complex.


serum
Patient
3
14
10
6
Precipitation (%)
100
75
50
25
0
0
20
40
60
80
100
Goat anti-(human IgG) (µl)


IgG
Patient
3
14
10
6
Precipitation (%)
100
75
50
25
0
0
20
40
60
80
Goat anti-(human IgG) (µl)

As the percentage precipitation effect observed did not seem to be related to the above trivial factors, other possibilities were investigated.

4.3.1 Heterogeneity of anti-(AChR) antibody subpopulations

One possible explanation for incomplete precipitation of [^{125}I]α-BGT - AChR is the absence of particular anti-(AChR) antibody subpopulations from the sera of certain myasthenic patients; coupled with heterogeneity of toxin binding components. This possibility was investigated by combining different sera together in an attempt to effect total precipitation of [^{125}I]α-BGT - AChR. The RIA for percentage precipitation of [^{125}I]α-BGT by excess myasthenic serum was performed in the usual way, but with one additional step:- after incubation of [^{125}I]α-BGT - AChR with a first serum for 2 hours, a second myasthenic serum was added for a further 2 hours incubation before addition of goat anti-(human IgG) antiserum. The results are shown in Table 13. Figure 27 shows a representative set of results obtained for addition of different sera to 1 patient who typically precipitated 50% of available [^{125}I]α-BGT labelled AChR. Overall, it can be seen that the addition of a "high-precipitating" to a "low-precipitating" serum did not increase the precipitation above that caused by the first addition alone. It is therefore evident that there is no additivity between the sera investigated.

4.3.2 [^{125}I]α-BGT - displacing antibodies

An alternative explanation for partial precipitation of [^{125}I]α-BGT labelled AChR involves the presence of antibodies which displace [^{125}I]α-BGT from [^{125}I]α-BGT labelled AChR complex, either

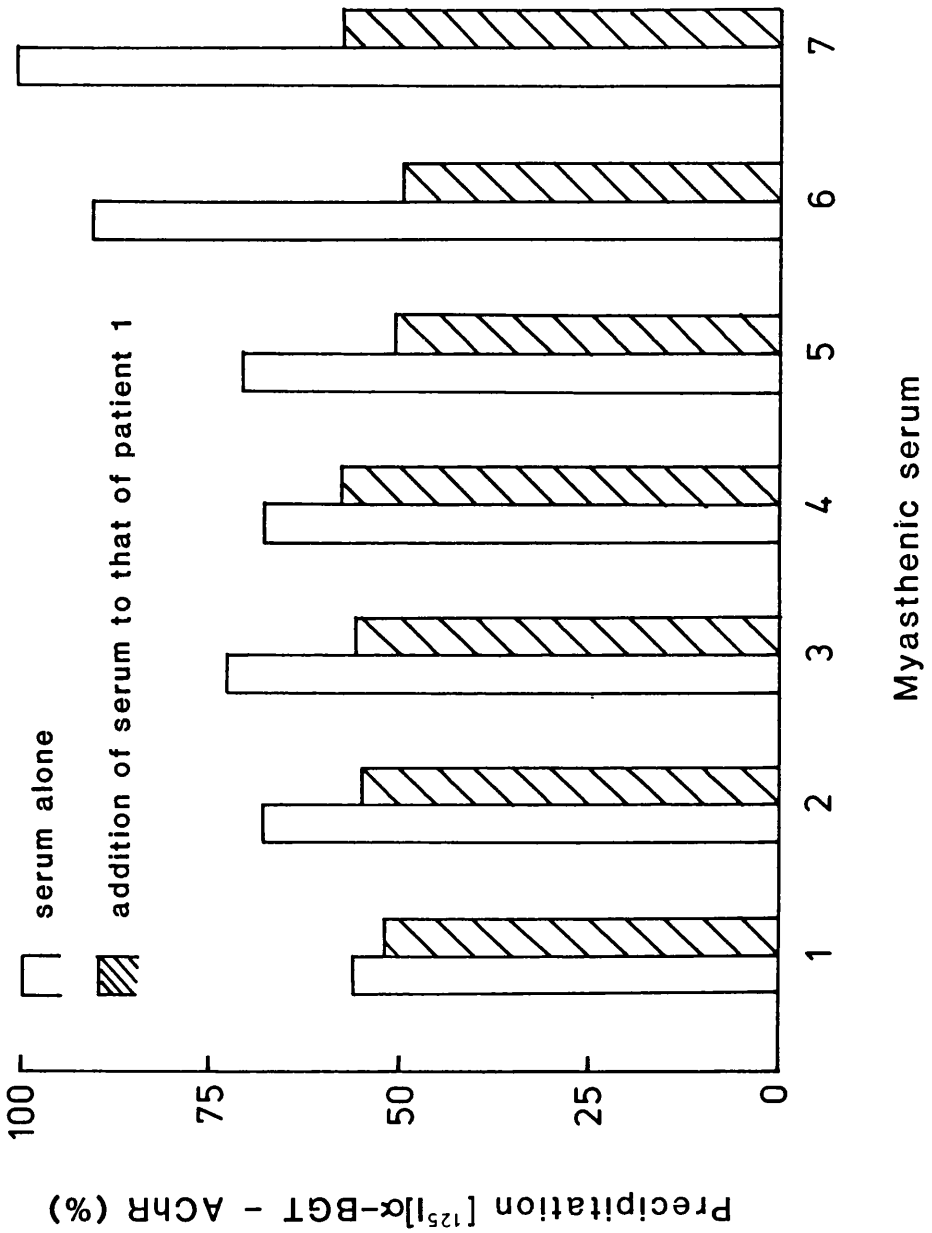
Table 13. The effect of combining different myasthenic sera on the percentage precipitation of [^{125}I]α-BGT - labelled AChR

Patient, first addition	Patient, second addition: N	6	14	15	10	8	11	3
N	0	69	74	79	81	83	91	100
6	57	53	55	56	58	52	51	59
14	68	64	59	59	58	54	63	60
15	73	74	79	75	78	74	83	76
10	68	66	67	68	67	62	62	65
8	71	64	67	76	65	66	71	73
11	92	75	82	87	82	85	97	97
3	102	82	87	96	91	86	101	98

Values are means of 4 determinations.

Figure 27. Partial precipitation of AChR by excess MG sera - illustration of the results obtained from a series of second additions to serum from that of patient 1

The radioimmunoassay for the percentage precipitation of [^{125}I]α-BGT labelled AChR by excess MG serum was performed in the usual way (see "Methods" section 7.3), but with one additional step:- After incubation of [^{125}I]α-BGT labelled AChR with the first myasthenic serum for 2 h, a second myasthenic serum was added for a further 2 h incubation before addition of goat anti-(human IgG) antiserum (see also Table 13).

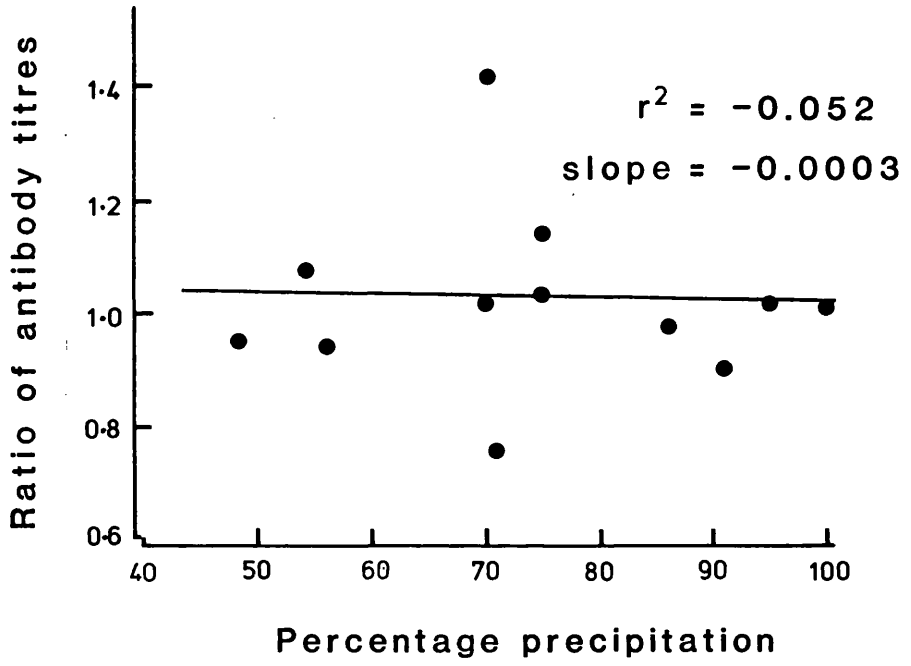

Precipitation [125I]α-BGT - AChR (%)
100
75
50
25
0
serum alone
addition of serum to that of patient 1
1
2
3
4
5
6
7
Myasthenic serum

by binding at or near the ACh binding site, or by binding elsewhere and promoting a conformational change. Attempts to demonstrate the presence of antibodies directed at the ACh binding site (anti-site antibodies) by the inhibition of [^{125}I]α-BGT binding to AChR following pre-incubation with myasthenic serum have given equivocal results (Fulpius, 1983). The relationship between inhibition of [^{125}I]α-BGT binding to AChR by myasthenic sera and percentage precipitation of [^{125}I]α-BGT labelled AChR in 12 patients is shown in Figure 28. It can be seen that sera from only a few patients actually inhibit the binding of [^{125}I]α-BGT to AChR, and that there is no direct correlation with the percentage precipitation of [^{125}I] α-BGT labelled AChR by sera from these patients.

One way of looking for possible toxin-releasing antibodies is to follow the time-dependent displacement of [^{125}I]α-BGT from [^{125}I] α-BGT labelled AChR in the presence of myasthenic sera.

[^{125}I]α-BGT - AChR was incubated with buffer, normal human serum, unlabelled -BGT or myasthenic serum and any displaced [^{125}I] α-BGT was separated from [^{125}I]α-BGT - AChR by gel filtration (see "Methods" section 11) over a period of time. The disappearance of radiolabel from the void volume peak and its appearance in the low MW peak was monitored. The results are shown in Figure 29 and Table 14. Although this method demonstrated both the release of radiolabel from the high MW peak and its appearance in the low MW peak, it was both slow and cumbersome, which precluded a more detailed study. For these reasons the separation of bound and free species was subsequently achieved by ion-exchange filtration on DEAE-cellulose filter discs (see "Methods" section 7.2). This has the disadvantage of measuring only the [^{125}I]α-BGT - AChR complex remaining, but this

Figure 28. Relationship between inhibition of [^{125}I]α-BGT binding to AChR by myasthenic serum and the percentage precipitation of [^{125}I]α-BGT labelled AChR by myasthenic serum



Inhibition of [^{125}I]α-BGT binding was calculated as the ratio of antibody titres $\frac{\text{titre a}}{\text{titre b}}$

where titre a was determined by incubation of myasthenic serum (5 µl) with AChR (0.5 nM) for 2 hours at 23°C followed by addition of [^{125}I]α-BGT (5 nM) for 2 hours at 23°C followed by precipitation with goat anti-(human IgG) antiserum (see "Methods" section 8) and titre b was determined by the standard radioimmunoassay procedure (see "Methods" section 8). The percentage precipitation of [^{125}I]α-BGT labelled AChR by excess myasthenic serum was determined as described (see "Results" section 4.2).

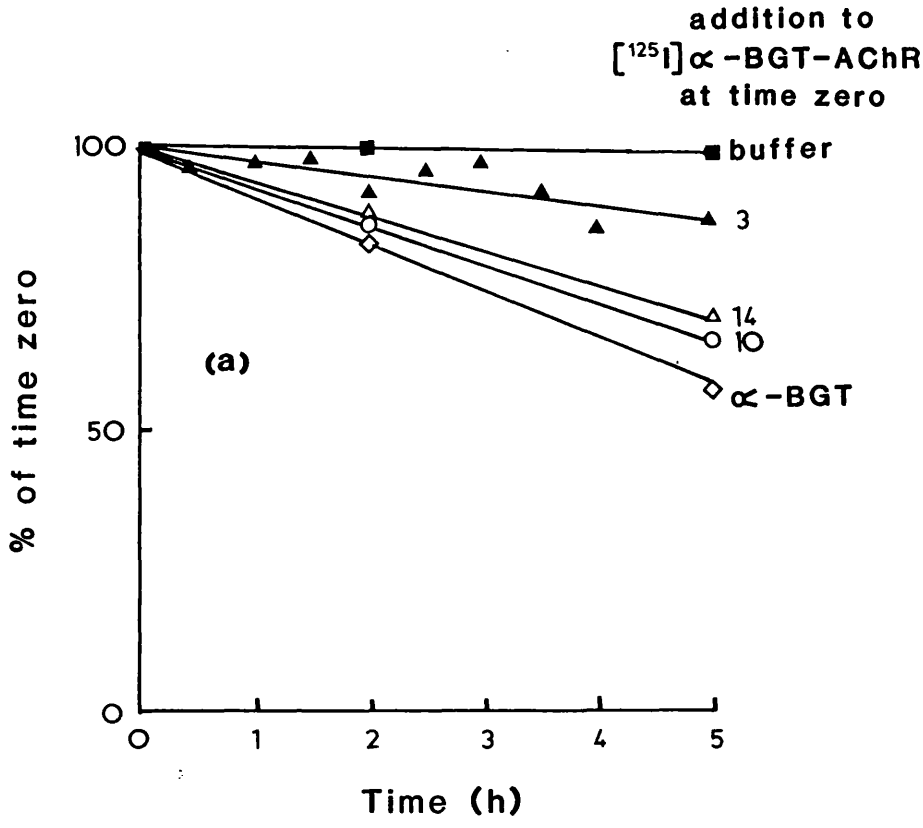
Figure 29. Displacement of [^{125}I]α-BGT from [^{125}I]α-BGT labelled AChR by myasthenic sera - data obtained from gel filtration chromatography

Duplicate samples [^{125}I]α-BGT-AChR (100 µl), labelled in the presence and absence of benzoquinonium chloride, were incubated with myasthenic sera, or with, as controls, buffer or unlabelled α-BGT (5 - 150 µl) for various times, up to 6 hours, after which the reaction was terminated by gel filtration on Sephadex G-75. Fractions were collected and aliquots counted for radioactivity. The amount of [^{125}I]α-BGT labelled AChR at each time point was expressed as a percentage of the amount present at zero time.

Each line represents the mean of 4 experiments, the points shown result from one typical experiment. Numbers refer to serum samples from different MG patients.

(a) disappearance of [^{125}I]α-BGT from [^{125}I]α-BGT labelled AChR in the void volume peak

(b) appearance of [^{125}I]α-BGT in the low molecular weight peak


addition to
[125I]α-BGT-AChR
at time zero
100
50
0
% of time zero
(a)
buffer
3
14
10
α-BGT
0
1
2
3
4
5
Time (h)

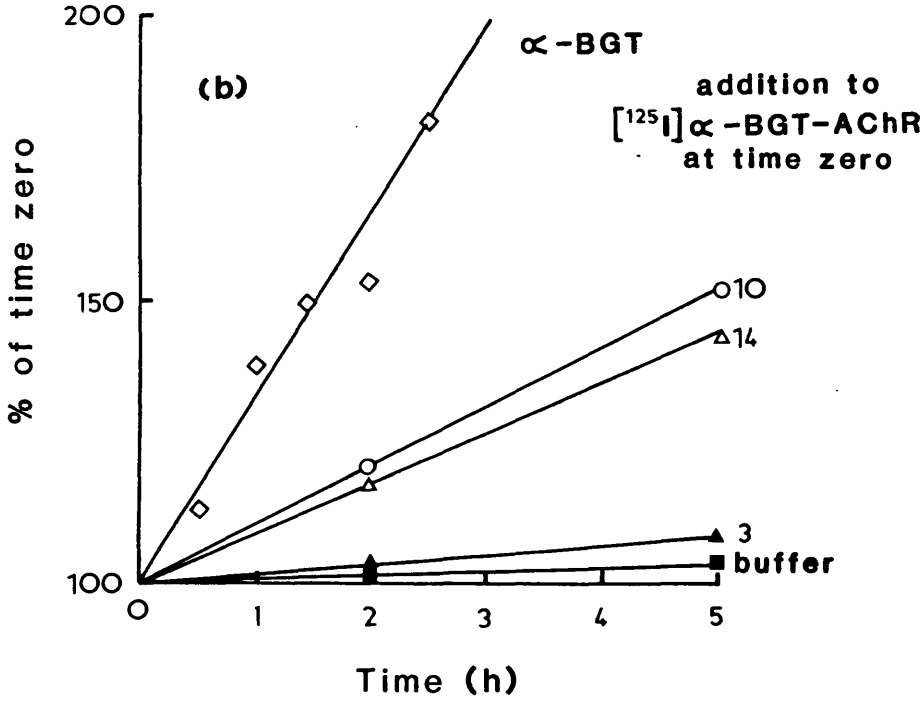

200
150
100
% of time zero
(b)
α-BGT
addition to
[125I]α-BGT-AChR
at time zero
10
14
3
buffer
0
1
2
3
4
5
Time (h)

Table 14. Displacement of [^{125}I]α-BGT from [^{125}I]α-BGT - labelled AChR : gel filtration

	Percentage [^{125}I]α-BGT - AChR remaining at time (h)		
Addition of	2	5	24
buffer	99	99	107
α-BGT	82	58	37
Patient 3	94	88	73
Patient 6	84	68	ND
Patient 14	82	70	ND

ND = not determined

Values are means of 4 determinations.

is compensated for by the advantages of speed, reproducibility and the opportunity of monitoring several samples in parallel, thus facilitating direct comparisons between different sera used. Figure 30 and Table 15 show the results obtained from this method.

The results show that some myasthenic sera were capable of accelerating the release of bound [^{125}I]α-BGT from its receptor complex. The extents to which this occurred showed an approximately inverse relationship with the ability of the sera to precipitate receptor (see "Results" section 4.3.1). Control experiments where [^{125}I]α-BGT labelled AChR complexes were incubated in the presence of "high-precipitating" sera, buffer or normal human serum showed comparatively very slow dissociation of [^{125}I]α-BGT from such complexes.

4.4 Inhibition Factor

In the RIA for anti-(AChR) antibodies, sera from most patients, when serially diluted, precipitate a corresponding amount of [^{125}I]α-BGT-AChR. However, there are some patients, approximately 20%, whose sera precipitate a higher amount of [^{125}I] -BGT-AChR complex than would be expected with increasing dilution. This suggested the presence, in some sera, of a factor which inhibited or protected the receptor-antibody interaction and, when diluted, allowed binding of more [^{125}I]α-BGT-AChR to antibody. This factor is referred to as the "inhibition factor".

At first it was thought that this observation may be related to the percentage precipitation of [^{125}I]α-BGT-AChR described in the previous section, as there seemed to be some correlation between the two effects (see Figure 31). However, extension of the original

Figure 30. Displacement of [^{125}I]α-BGT from [^{125}I]α-BGT labelled AChR by myasthenic sera - data obtained from DEAE-cellulose disc filtration

Duplicate samples [^{125}I]α-BGT-AChR (100 µl), labelled in the presence and absence of benzoquinonium chloride, were incubated with myasthenic sera, or with, as controls, buffer, normal serum or unlabelled α-BGT (5 - 150 µl) for various times, up to 24 h, after which the reaction was terminated by ion-exchange filtration on DEAE-cellulose filter discs (see "Methods" section 7.2). The amount of [^{125}I]α-BGT labelled AChR at each time point was expressed as a percentage of the amount present at zero time. The results shown are the mean of 4 determinations. Numbers refer to serum samples from different myasthenic patients.

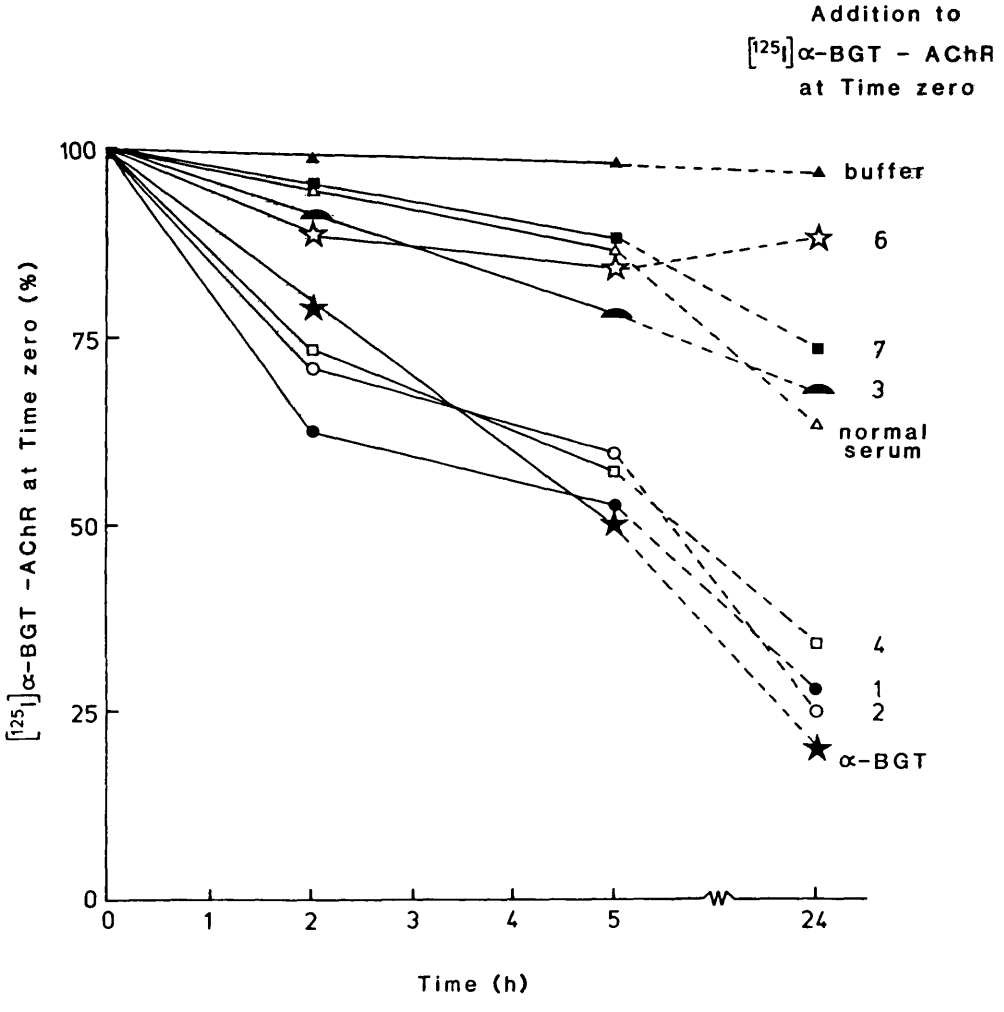

Addition to
[125I]α-BGT - AChR
at Time zero
buffer
6
7
3
normal serum
4
1
2
α-BGT
100
75
50
25
0
0
1
2
3
4
5
24
[125I]α-BGT -AChR at Time zero (%)
Time (h)

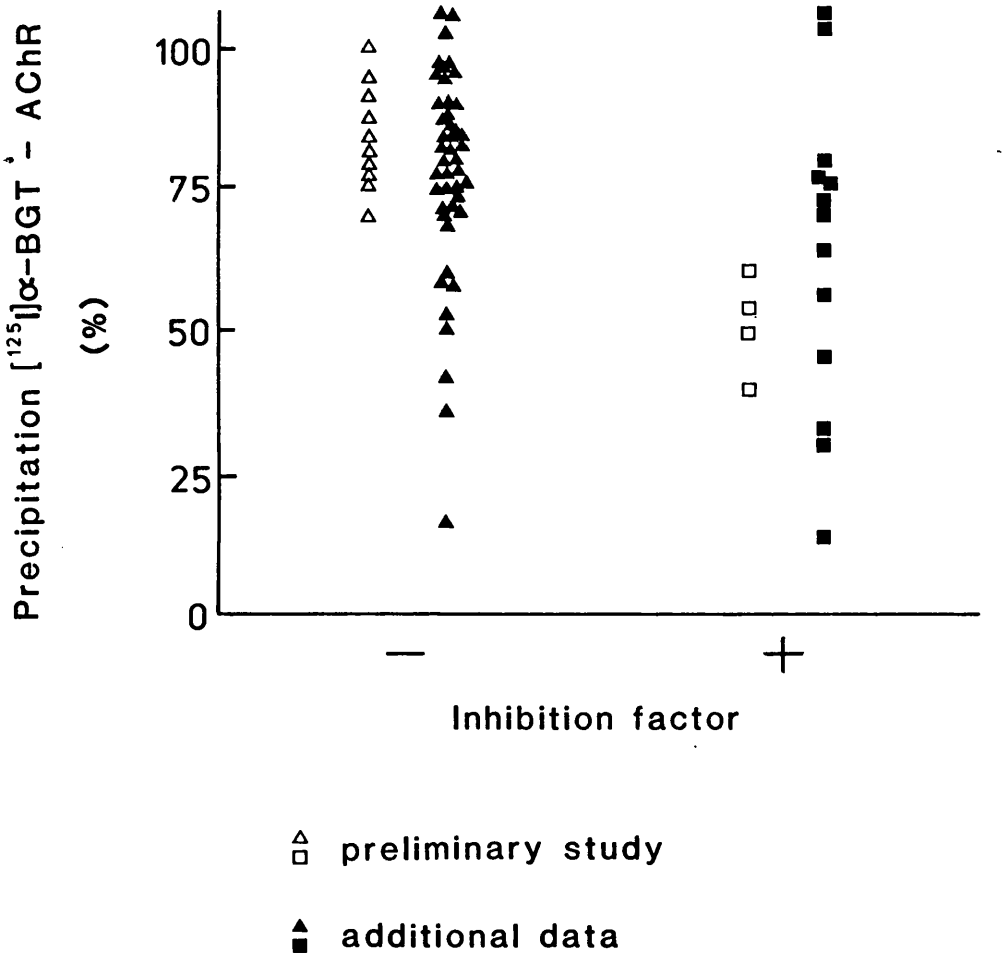
Table 15. Displacement of [^{125}I]α-BGT from [^{125}I]α-BGT - labelled AChR : DEAE-cellulose filtration

Addition of	Percentage [^{125}I]α-BGT - AChR remaining at time (h)		
	2	5	24
buffer	94	108	97
α-BGT	77	53	20
normal serum	95	86	63
Patient 6	63	52	28
" 14	71	60	25
" 15	90	78	70
" 10	72	58	34
" 8	ND	ND	ND
" 11	88	90	92
" 3	93	88	73

ND = Not determined

Values are means of 3 determinations

Figure 31. Relationship between percentage precipitation of [^{125}I]α-BGT labelled AChR and the presence or absence of "inhibition factor" in myasthenic sera



preliminary study (see Figure 31) failed to substantiate the initial relationship. The nature of the inhibition factor was then investigated by several approaches:-

1. Increasing volumes of myasthenic sera were used to precipitate [^{125}I]α-BGT labelled AChR complexes, but this did not remove the "inhibition factor" effect, neither did increasing the amount of goat anti-(human IgG) antiserum used to precipitate the labelled complexes. These results suggest that the "inhibition factor" effect is not caused by insufficient or excess myasthenic serum or second antibody.
2. Serum from 1 MG patient with the "inhibition factor" was fractionated on a Sephacryl S-300 column. The IgG fractions obtained from this fractionation showed no "inhibition factor" effect. Adding the putative inhibitory fractions back to such IgG in the radioimmunoassay failed to show the "inhibition factor" effect, possibly because the fractions were too dilute after processing.
3. Serum from 4 MG patients, two with, and two without, the "inhibition factor", were passed through a column of α-toxin coupled to Sepharose 4-B which had been incubated with detergent extract of adult human AChR. The eluant was then added back to myasthenic IgGs in the radioimmunoassay, but no "inhibition factor" effect was observed, again possibly resulting from dilution of the samples during processing.
4. Dialysis of sera containing the "inhibition factor" eliminated its effect in 1 out of 2 sera tested. This equivocal result may indicate a molecular weight of around 8000 for the component but more work is obviously necessary to clarify this.

5. Interaction of detergent extracts of human adult and foetal AChR with [^{125}I]α-BGT

It has been previously shown (see "Results" section 4.2) that anti-(AChR) antibody titres measured using EJ-rat AChR as antigen in the RIA are higher than if J-rat AChR is used, implying the presence of antigenic determinants on EJ-rat AChR which are recognized uniquely by myasthenic sera. Reports in the literature of the similarity of EJ- and embryonic AChR have led to the hypothesis that embryonic AChR may represent the autoimmunogen in MG. As the study of rat AChR in this way can only provide limited information about the human system, this section of Results investigates the binding of human foetal AChR with [^{125}I]α-BGT by immunohistochemical and kinetic methods and the binding of [^{125}I]α-BGT labelled foetal AChR with anti-(AChR) antibodies in myasthenic sera. Concurrently, these interactions are compared with those of human adult AChR. Foetuses were obtained from prostaglandin-induced terminations, and were 13 - 22 weeks gestation. In order to obtain sufficient foetal muscle to give adequate yields of AChR (especially for purification) for study, it was necessary to pool muscle from several foetuses of different ages. It was necessary, therefore, to gain some indication as to whether the AChR undergoes major developmental changes over the age range used. Detailed analysis was clearly impossible, but the age-dependence of α-BGT binding sites was monitored as a possible pointer towards receptor variation.

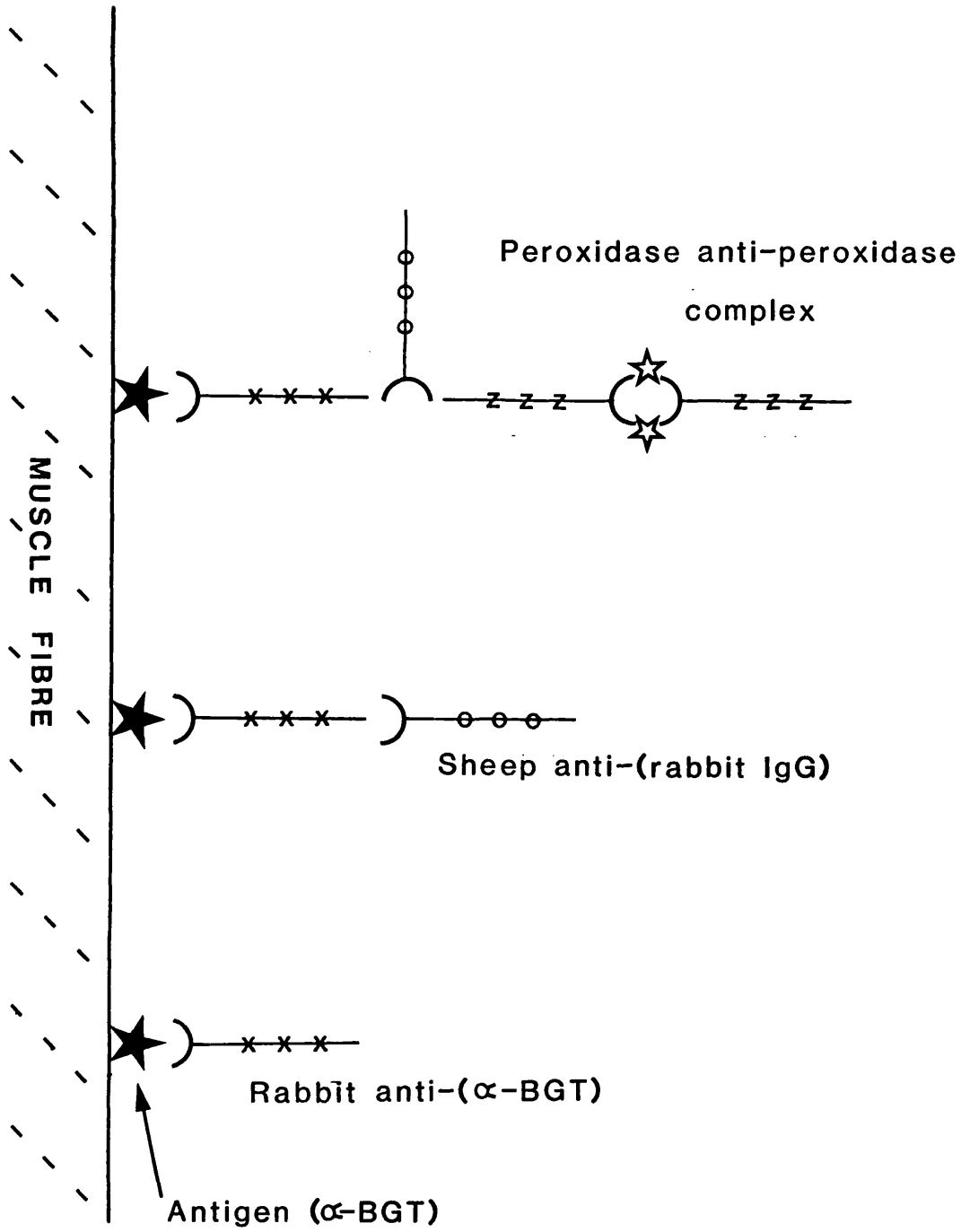
5.1 Immunohistochemical studies on human adult and foetal skeletal muscle

Preliminary attempts were made to visualize synapses in human foetal and adult skeletal muscle by staining α-BGT binding sites with the 'PAP' technique, by fluorescein conjugated α-BGT staining and by AChE staining, in order to try to distinguish between innervated and dennervated tissue.

5.1.1 Staining by the 'PAP' technique

Tissue sections from human foetal and adult skeletal muscle were prepared (see "Methods" sections 13.1 & 13.2) and stained with α-BGT by using the 'PAP' technique (see "Methods" section 13.3), a schematic diagram of which is given in Figure 32, showing the different stages of the staining procedure. Figures 33 - 38 show the staining of adult and foetal (12 & 18 weeks gestation) tissue by using this method. Apart from the controls in which normal rabbit serum replaced the anti-(α-BGT) antiserum, all sections were uniformly stained, irrespective of the inclusion of excess ligand to block α-BGT binding, or whether buffer was substituted in place of α-BGT in the first incubation step. This suggested that rabbit anti-(α-BGT) antiserum was binding non-specifically to the tissue types. For this reason, 2 absorption methods were carried out, using foetal tissue (18 weeks gestation) only, in order to attempt to reduce this heavy non-specific staining (see "Methods" section 13.4). The first absorption, against normal human eythrocytes gave no reduction in the background staining (results not shown). After the second absorption, against muscle extract, the rabbit anti-(α-BGT) antiserum retained 90% of its antigenic activity, as measured by RIA

Figure 32. Schematic outline of the 'PAP' method



Figures 33 - 38. Staining of human adult and foetal muscle sections with α-BGT using the 'PAP' technique (see "Methods" section 13.3)

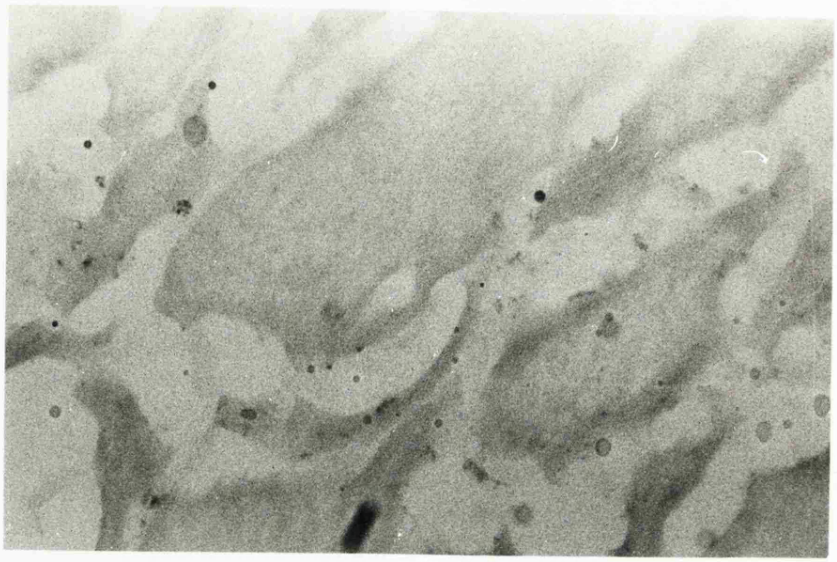
Figure 33. Staining of longitudinal sections of adult muscle

(A) The sections were incubated with α-BGT, rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(B) The sections were incubated with α-BGT, normal rabbit serum, sheep anti-(rabbit IgG) antiserum and PAP complex.

Magnification x 150

A



B

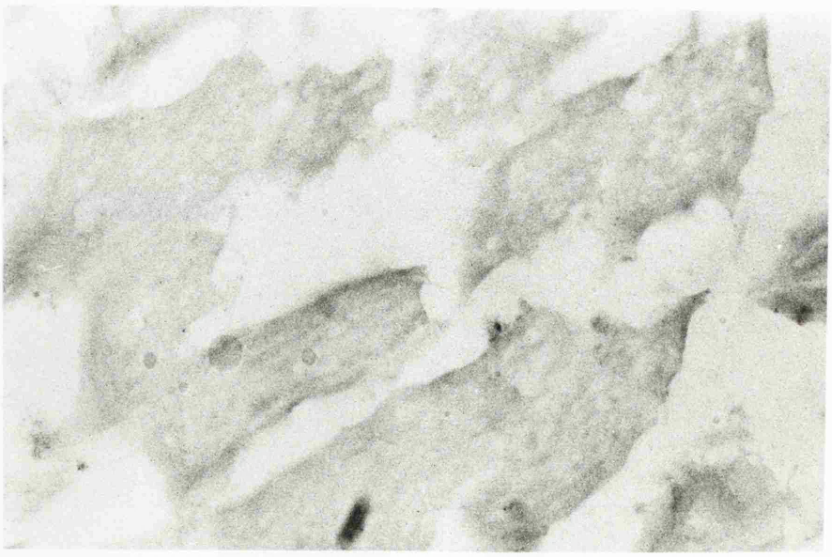


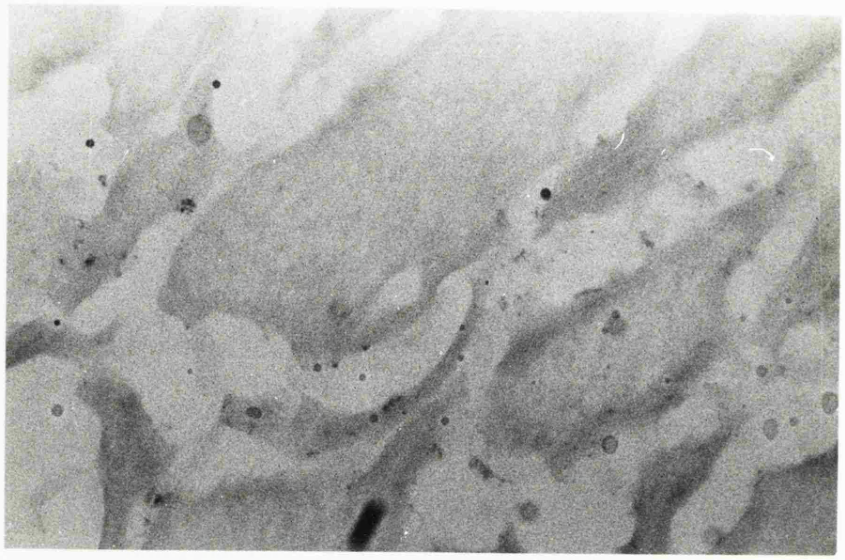
Figure 33. Staining of longitudinal sections of human adult muscle

(C) The sections were incubated with α-BGT following pre-incubation with d-tubocurarine, then with rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(D) The sections were incubated with buffer, rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

Magnification x 150

C



D

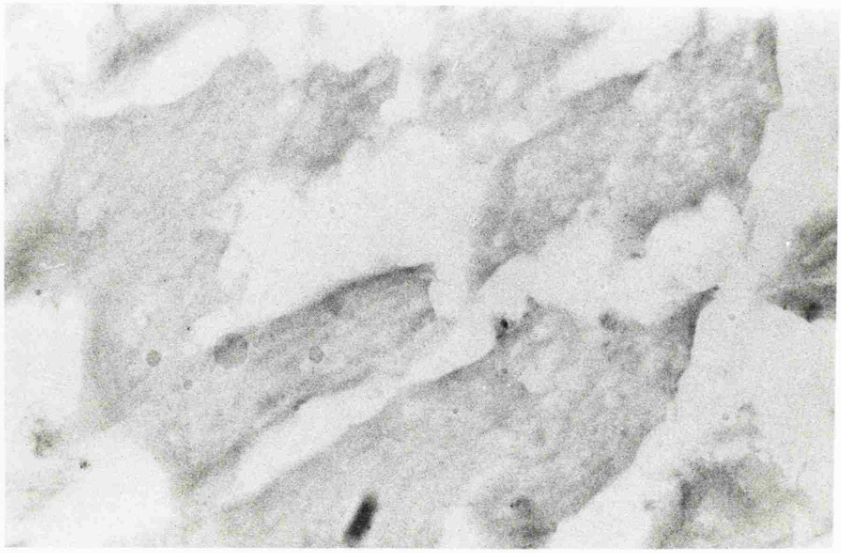


Figure 34. Staining of longitudinal sections of foetal (large) muscle

(A) The sections were incubated with α-BGT, rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(B) The sections were incubated with α-BGT, normal rabbit serum, sheep anti-(rabbit IgG) antiserum and PAP complex.

Magnification x 150

A

B

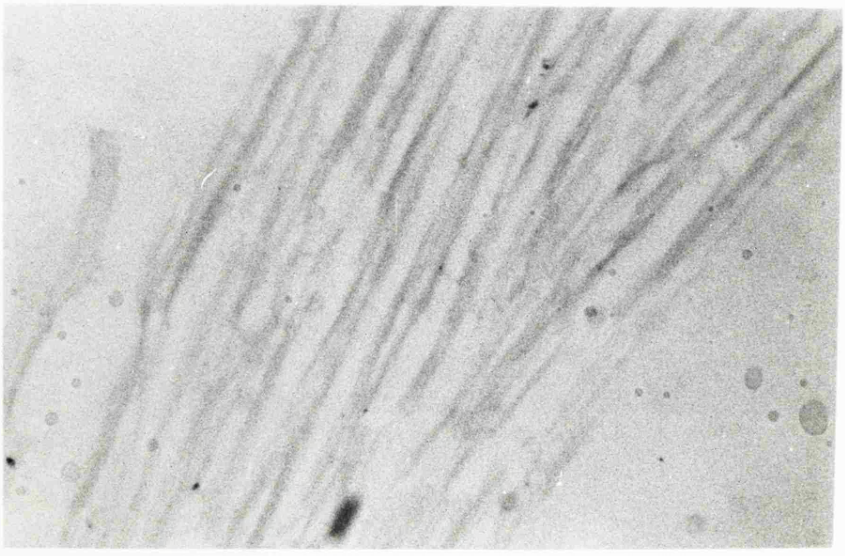


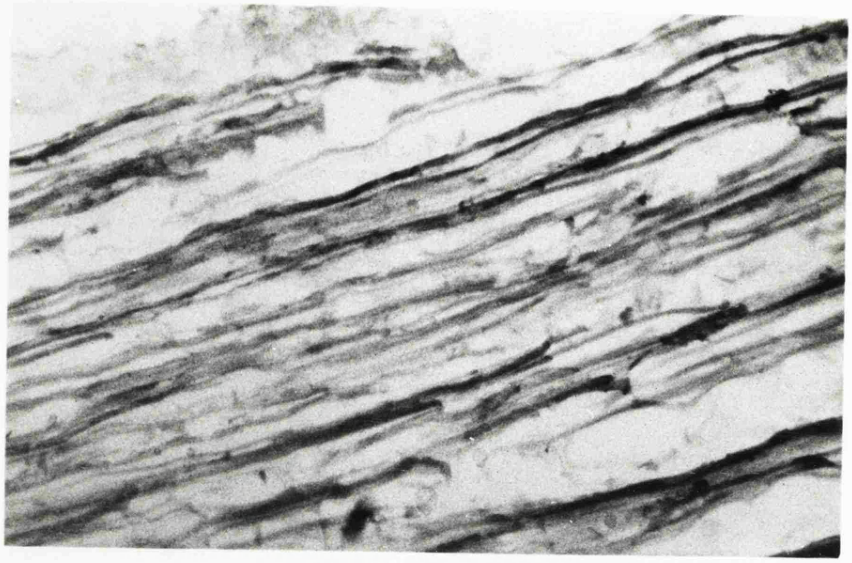
Figure 34. Staining of longitudinal sections of foetal (large) muscle

(C) The sections were incubated with α-BGT following pre-incubation with d-tubocurarine, then with rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(D) The sections were incubated with buffer, rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

Magnification x 150

C



D

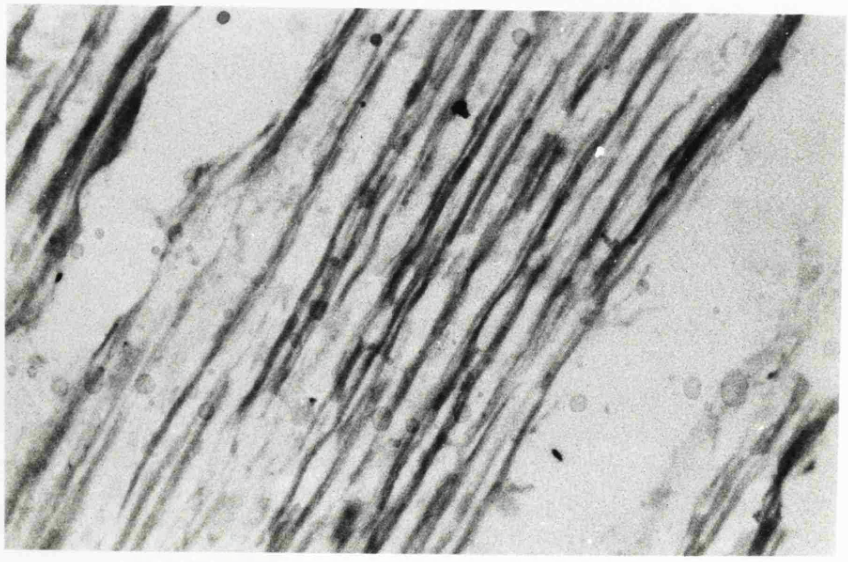


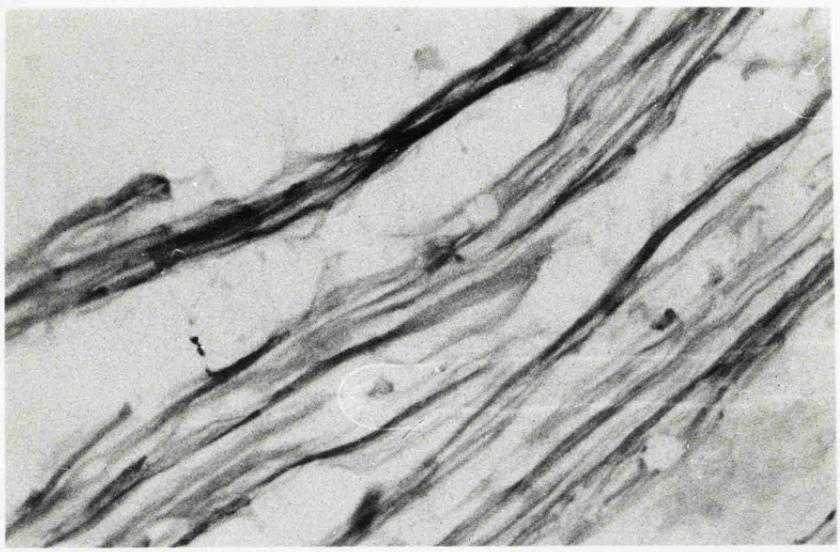
Figure 35. Staining of longitudinal sections of foetal (small) muscle

(A) The sections were incubated with α-BGT, rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(B) The sections were incubated with α-BGT, normal rabbit serum, sheep anti-(rabbit IgG) antiserum and PAP complex.

Magnification x 150

A



B

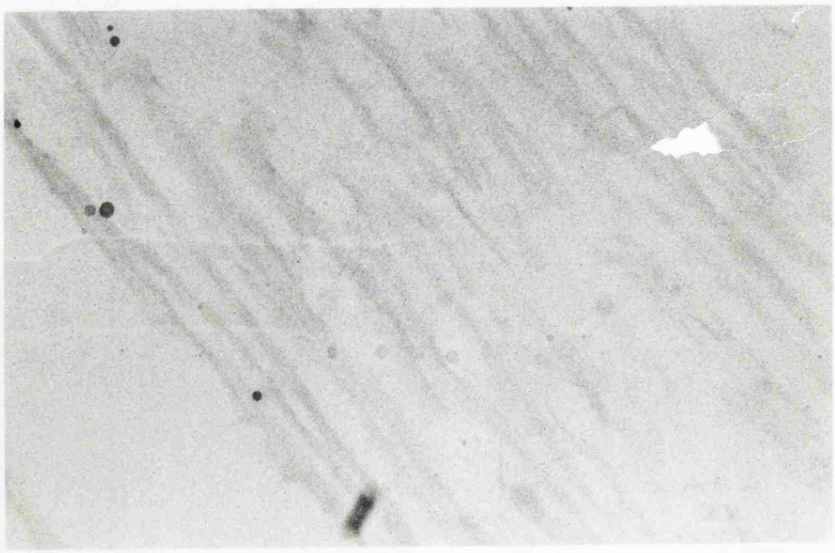


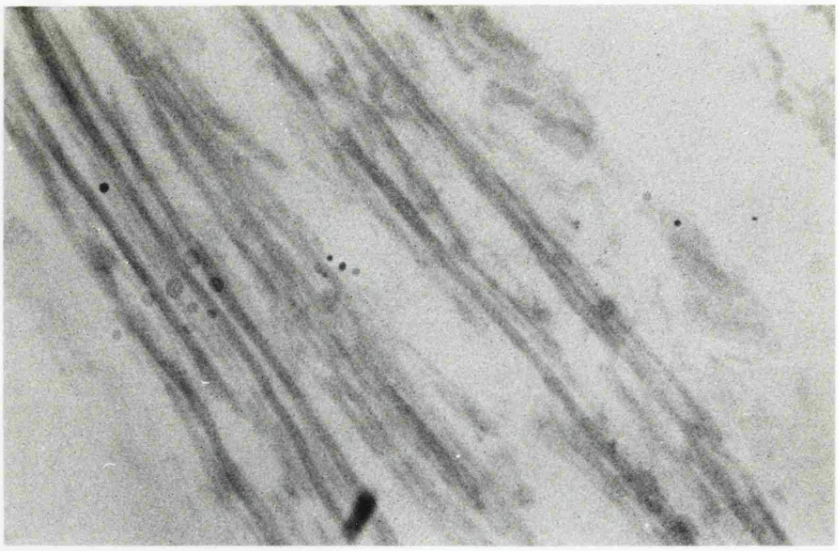
Figure 35. Staining of longitudinal sections of foetal (small) muscle

(C) The sections were incubated with α-BGT following pre-incubation with d-tubocurarine, then with rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(D) The sections were incubated with buffer, rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

Magnification x 150

C



D

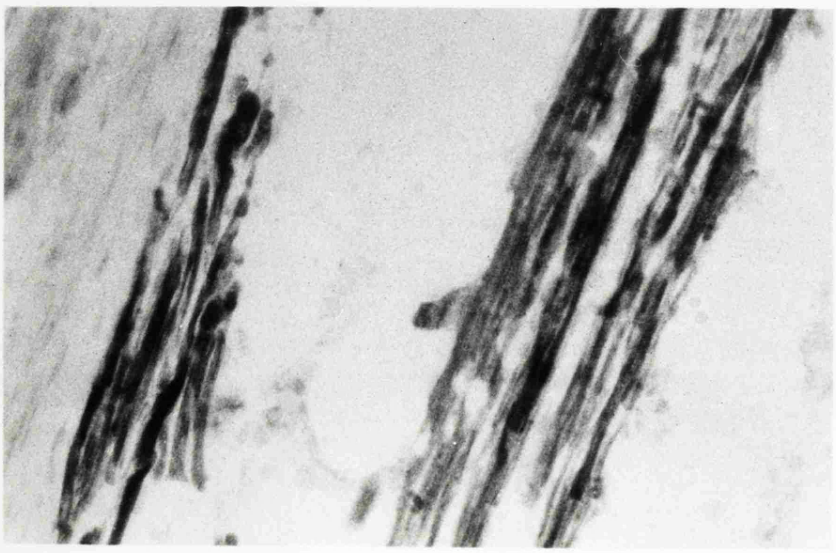


Figure 36. Staining of foetal (large) muscle sections after absorption of rabbit anti-(α-BGT) against human muscle

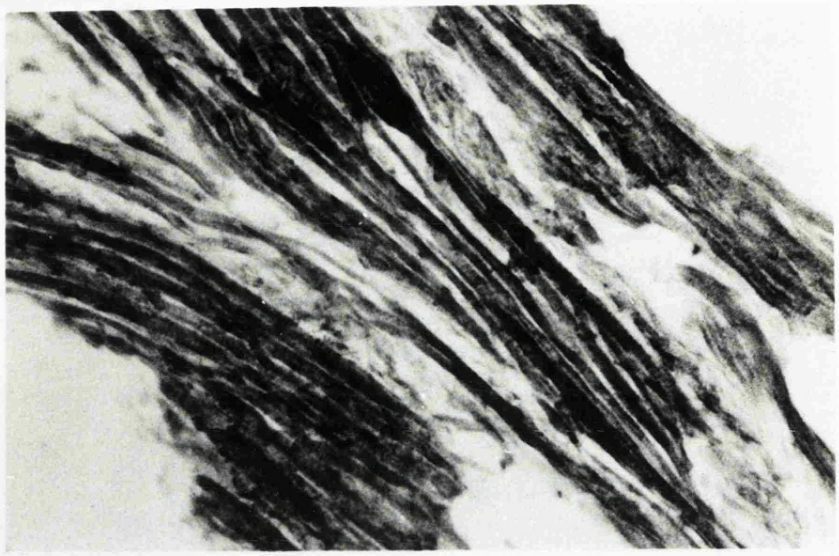
Sections were incubated with α-BGT, rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(A) Non-absorbed rabbit anti-(α-BGT) antiserum

(B) Rabbit anti-(α-BGT) antiserum absorbed against human muscle (see "Methods" section 13.4(ii)).

Magnification x 150

A



B

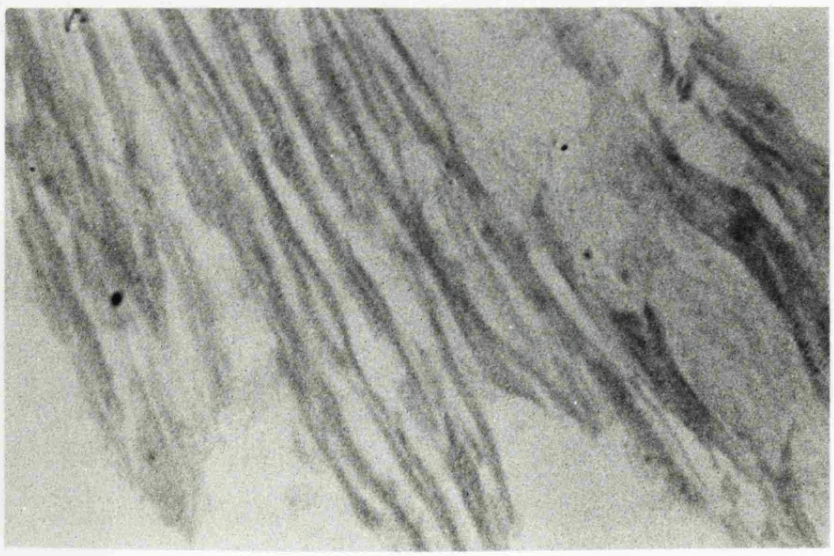


Figure 37. Staining of foetal (large) muscle sections after absorption of rabbit anti-(α-BGT) against human muscle

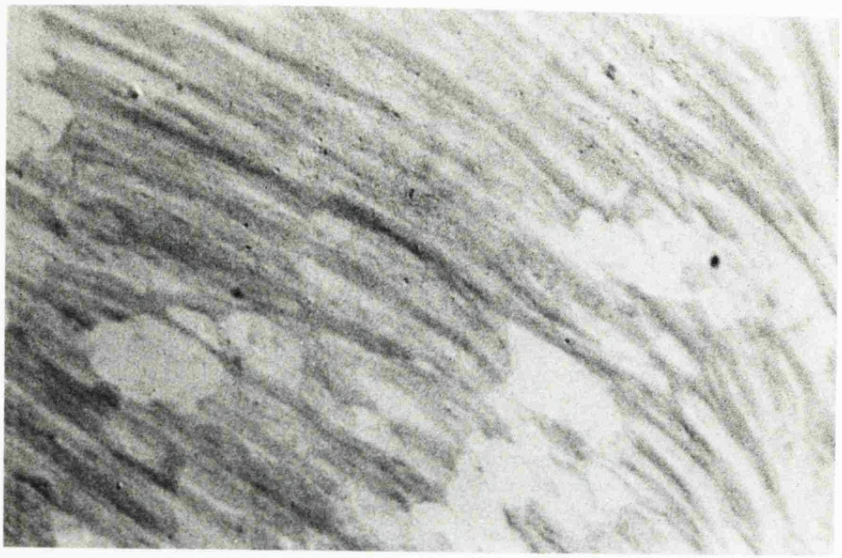
Sections were incubated with α-BGT, following pre-incubation with d-tubocurarine, then with rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(A) Non-absorbed rabbit anti-(α-BGT) antiserum

(B) Rabbit anti-(α-BGT) antiserum absorbed against human muscle (see "Methods" section 13.4(ii)).

Magnification x 150

A



B

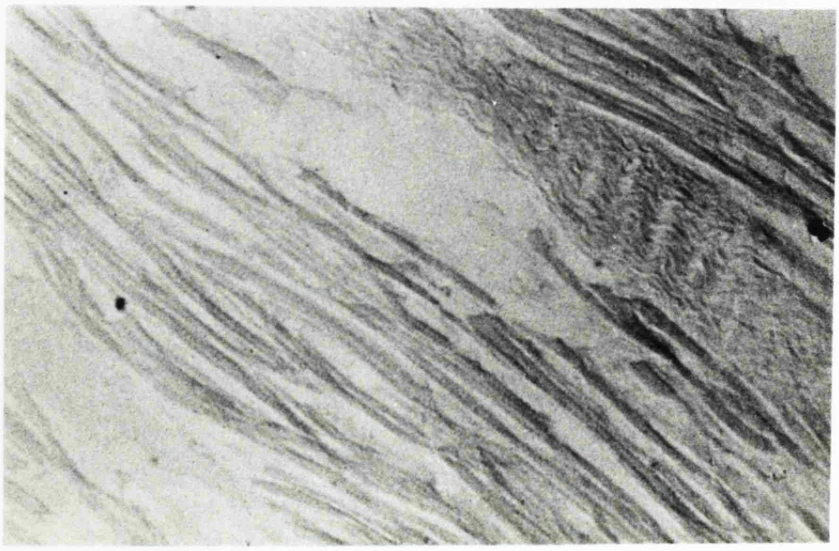


Figure 38. Staining of foetal (large) muscle sections after absorption of rabbit anti-(α-BGT) against human muscle

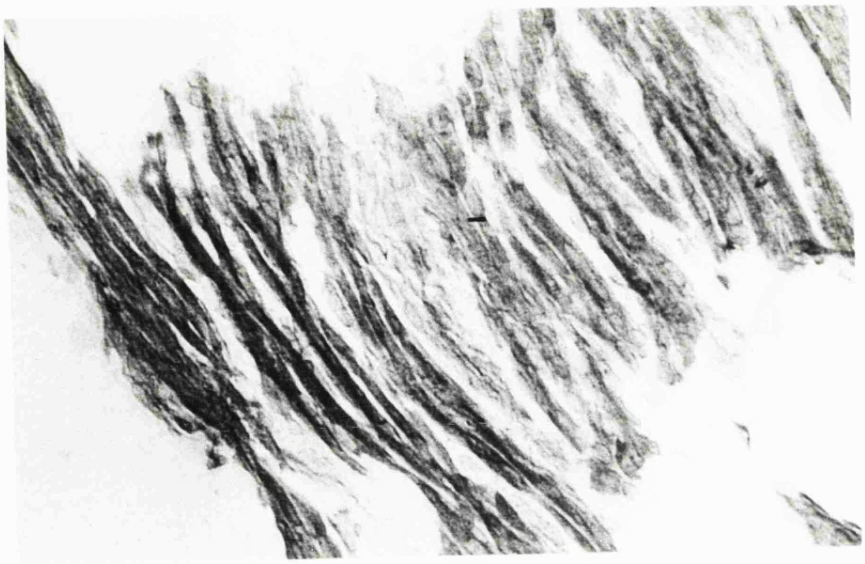
Sections were not incubated with α-BGT, but with buffer, then with rabbit anti-(α-BGT) antiserum, sheep anti-(rabbit IgG) antiserum and PAP complex

(A) Non-absorbed rabbit anti-(α-BGT) antiserum

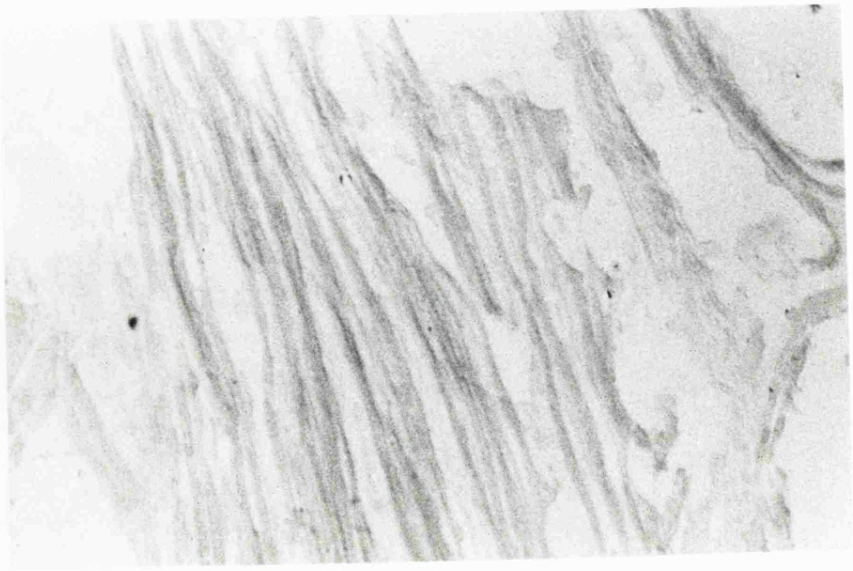
(B) Rabbit anti-(α-BGT) antiserum absorbed against human muscle (see "Methods" section 13.4(ii)).

Magnification x 150

A



B



(see "Methods" section 9). The amount of non-specific staining was reduced (Figures 36 - 38), but there was still not enough difference between the staining patterns of the tissue types to draw any conclusions concerning the distribution of end-plates in these muscle sections.

5.1.2 Staining with fluorescein-conjugated α-BGT

Muscle fibres were teased from human adult and foetal (18 weeks gestation) muscle and stained with fluorescein-conjugated α-BGT as described (see "Methods" section 13.5). Foetal muscle showed uniform weak fluorescence along rows of myotubes (results not shown). Adult muscle showed intense diffuse fluorescent staining which did not appear to be localized at end-plates, and which was abolished in fibres pre-incubated with unlabelled α-BGT (see Figure 39). Areas of fluorescence corresponded with areas which were counterstained with methyl green and viewed by transmitted light.

5.1.3 Staining for AChE

In contrast to the results obtained for fluorescein staining, adult muscle fibres showed no staining for AChE, whereas foetal muscle fibres showed staining along the whole length of muscle fibres; this was abolished in the presence of neostigmine (see Figure 40).

As these studies were largely inconclusive, α-BGT and anti-(AChR) antibody binding were examined in solubilized preparations of adult and foetal muscle.

Figure 39. Staining of teased fibres from human adult muscle with fluorescein-conjugated α-BGT

Fibres were stained as described in the "Methods" section 13.5.

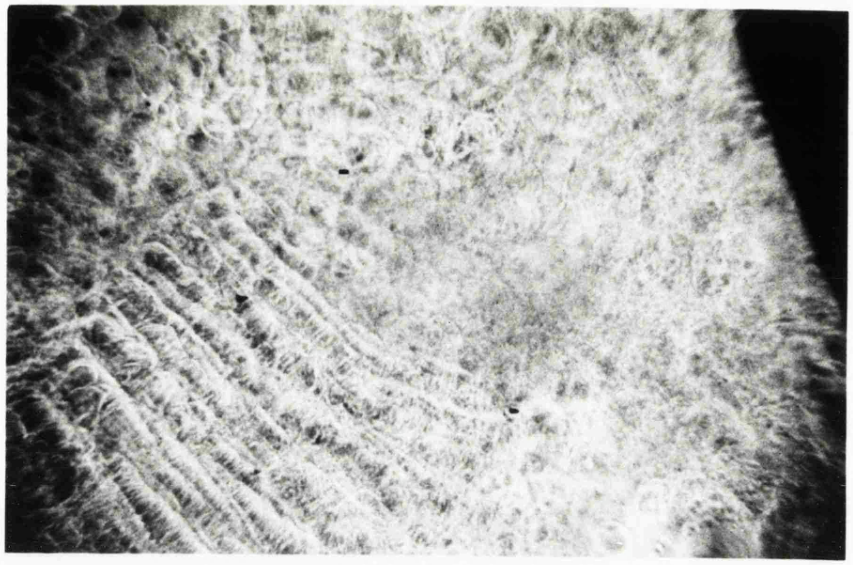
(A) Transmitted light

(B) Fluorescence staining

Magnification x 150

When fibres were pre-incubated with unlabelled α-BGT before the staining procedure, all fluoresence was abolished.

A



B

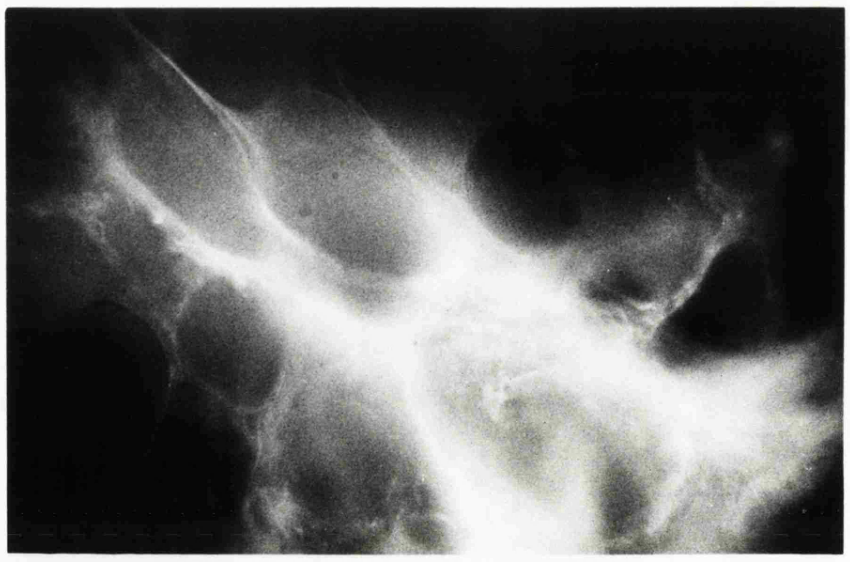


Figure 40. Staining of teased fibres from human foetal muscle for AChE activity

Fibres were stained as described in the "Methods" section 13.6.

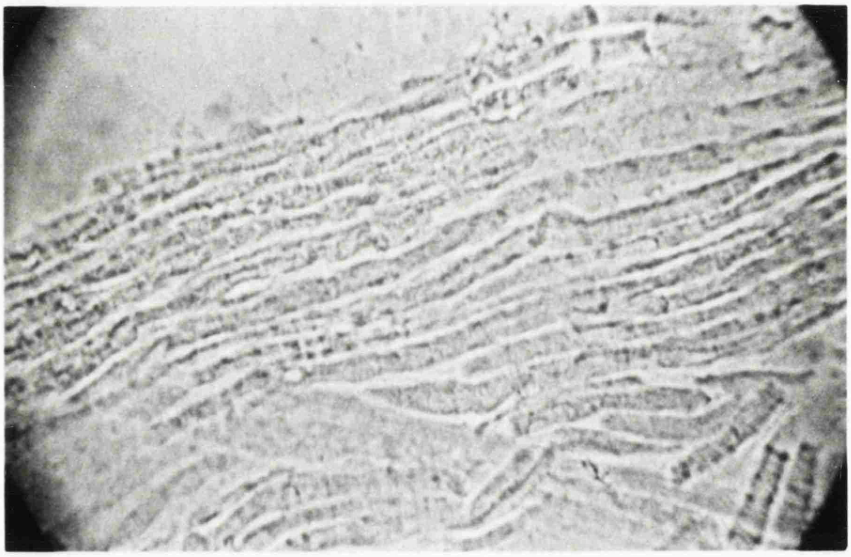
(A) Magnification x 150

(B) Magnification x 600

(C) Teased fibres stained after pre-incubation with neostigmine, magnification x 150

A

B



C

5.2 Determination of [^{125}I]α-BGT binding sites versus foetal age

Levels of [^{125}I]α-BGT binding in "crude" detergent extracts from individual foetuses, aged 13 - 19 weeks gestation, were compared (see Figure 41). Foetal age was assessed by crown-rump length (Arey, 1954). The concentration of specific α-BGT binding sites varies between 0.6 - 1.8 pmol/g wet weight of tissue over the age range studied, and this agrees well with the range for adult muscle. There was no correlation between the yield of [^{125}I]α-BGT binding sites and foetal age.

5.3 Determination of rate constants for [^{125}I]α-BGT binding to human foetal AChR and comparison with human adult AChR

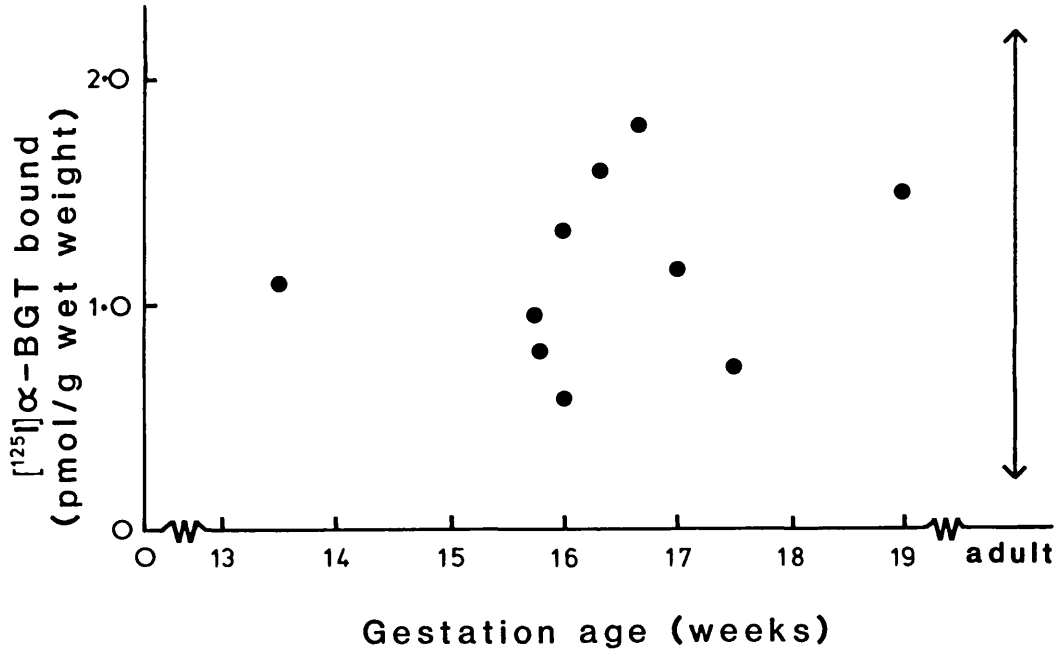
The interaction of [^{125}I]α-BGT with detergent extracts of human adult and foetal AChR were compared. Although the ammonium sulphate assay was usually the preferred method for determination of [^{125}I]α-BGT equilibrium binding in detergent extracts of AChR (see "Methods" section 7.1), it was found to be unsuitable for the rapid termination of binding necessary for kinetic determinations of association and dissociation rate constants (see "Results" section 3.1). Therefore, the DEAE-cellulose filtration assay (see "Methods" section 7.2) was used instead, with high activity extracts which were diluted in order to minimize adsorption of non-receptor proteins to the filter discs. From the law of mass action:-

$$R + L \rightleftharpoons RL \qquad \ldots\ldots.1$$

The association (k_{+1}) and dissociation (k_{-1}) rate constants can be defined in the following equation:-

$$\frac{d(RL)}{dt} = k_{+1}[R][L] - k_{-1}[RL] \qquad \ldots\ldots.2$$

Figure 41. Determination of [^{125}I]α-BGT binding sites versus foetal age



Triplicate samples (100 µl) of serial two-fold dilutions of detergent extracts of foetal skeletal muscle from individual foetuses were incubated with 5 nM [^{125}I]α-BGT in the presence and absence of a large molar excess (10^{-3} M) of benzoquinonium chloride, followed by precipitation with ammonium sulphate at 4°C for 16 h. Bound radiolabel was recovered by filtration on glass fibre filter discs as described in the "Methods" section 7.1.

5.3.1 Association Rate Constant (k_{+1})

The association rates for the binding of [^{125}I]α-BGT to detergent extracts of both foetal and adult AChRs were determined by 2 methods:-

(a) Second order kinetics

For the second order treatment of kinetic data the initial rate of binding is considered, ie. the bound concentration is assumed to be so low that $k_{-1}[RL]$ is essentially zero. By manipulation and integration of equation 2, the integrated second order rate equation is given as:-

$$\ln \frac{B_e(L_T - B)}{L_T(B_e - B)} = k_{+1}\, t\, (L_T - B_e) \qquad \ldots\ldots.3$$

where:- B_e = maximum concentration of binding sites

B = concentration bound at time t

L_T = total ligand concentration

t = time

k_{+1} = association rate constant

Figure 42 shows representative sets of progress curves for the reaction of [^{125}I]α-BGT with human adult and foetal AChR and Figure 43 the second order plots obtained from this data. The rate constants so obtained are given in Table 16(a).

(b) Pseudo first order kinetics

This method takes into account the reverse reaction ($k_{-1}[RL]$), but assumes that the concentration of radioactive ligand is constant. If the reaction is allowed to proceed to equilibrium, it can be shown that the bound concentration (B) at any prior time is related to the equilibrium concentration (B_e) by the equation:-

Figure 42. Time course of specific binding of [^{125}I]α-BGT at increasing [^{125}I]α-BGT concentrations

Samples of detergent extracts of adult or foetal AChR (100 µl) were incubated with increasing concentrations of [^{125}I]α-BGT in the presence and absence of a large molar excess of unlabelled α-BGT for various time intervals. Binding was stopped by the addition of cold toxin binding assay buffer and rapid filtration as described in the "Methods" section 7.2.

△ 1. 0.66 nM [^{125}I]α-BGT

□ 2. 1.32 nM [^{125}I]α-BGT

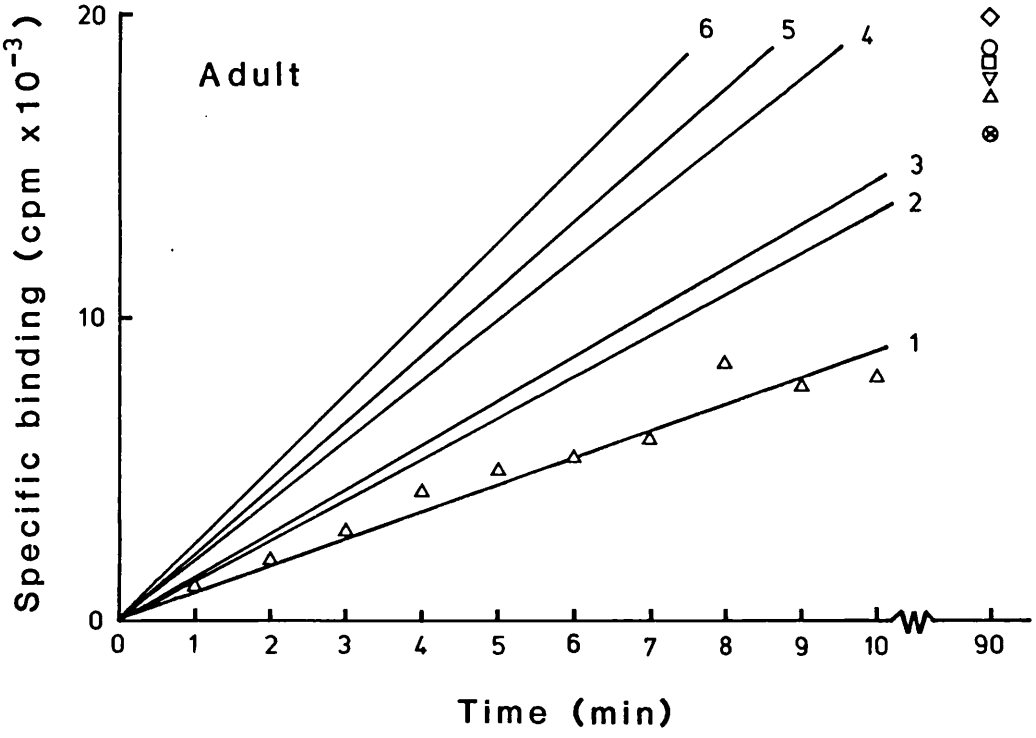
○ 3. 1.98 nM [^{125}I]α-BGT

▽ 4. 2.64 nM [^{125}I]α-BGT

⊗ 5. 3.30 nM [^{125}I]α-BGT

◇ 6. 3.96 nM [^{125}I]α-BGT

The amount of radiolabel bound at equilibrium (90 min) was taken as 100%. Points are means of duplicate determinations.


Adult
Specific binding (cpm x10^{-3})
20
10
0
0 1 2 3 4 5 6 7 8 9 10 90
Time (min)
6
5
4
3
2
1

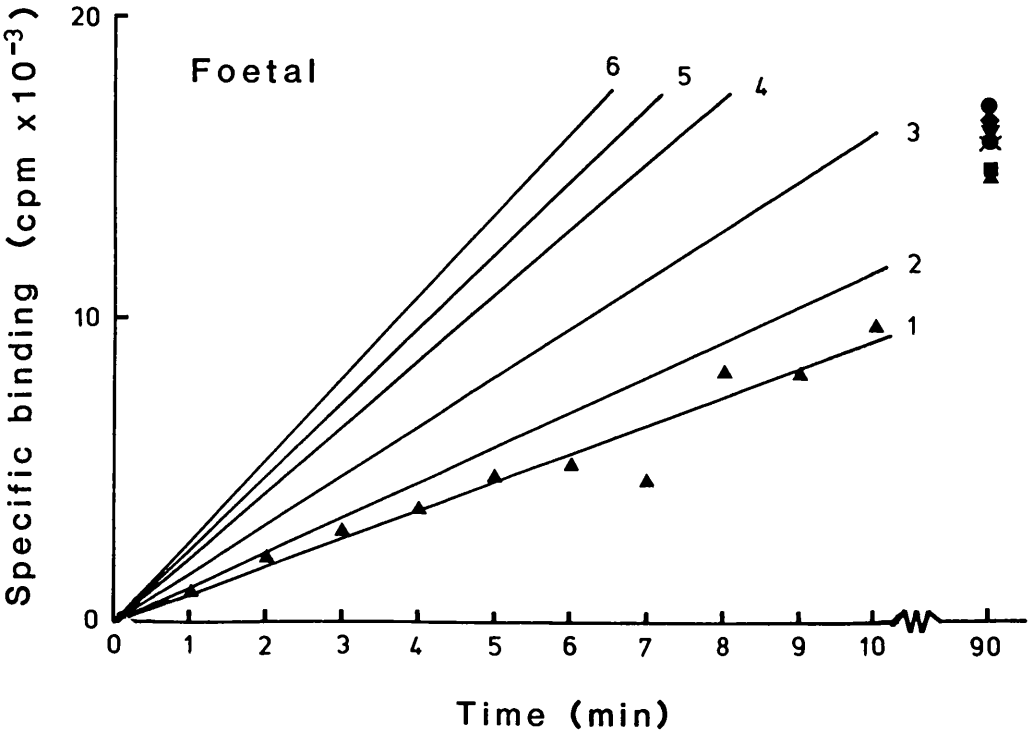

Foetal
Specific binding (cpm x10^{-3})
20
10
0
0 1 2 3 4 5 6 7 8 9 10 90
Time (min)
6
5
4
3
2
1

Figure 43. Second order treatment of binding data in Figure 42.

The slopes of the lines give values for the association rates at the different [^{125}I]α-BGT concentrations used

Adult:- 1. 17.30×10^5 $M^{-1}s^{-1}$

2. 12.70×10^5 $M^{-1}s^{-1}$

3. 9.81×10^5 $M^{-1}s^{-1}$

4. 8.43×10^5 $M^{-1}s^{-1}$

5. 7.73×10^5 $M^{-1}s^{-1}$

6. 7.61×10^5 $M^{-1}s^{-1}$

Foetal:- 1. 15.30×10^5 $M^{-1}s^{-1}$

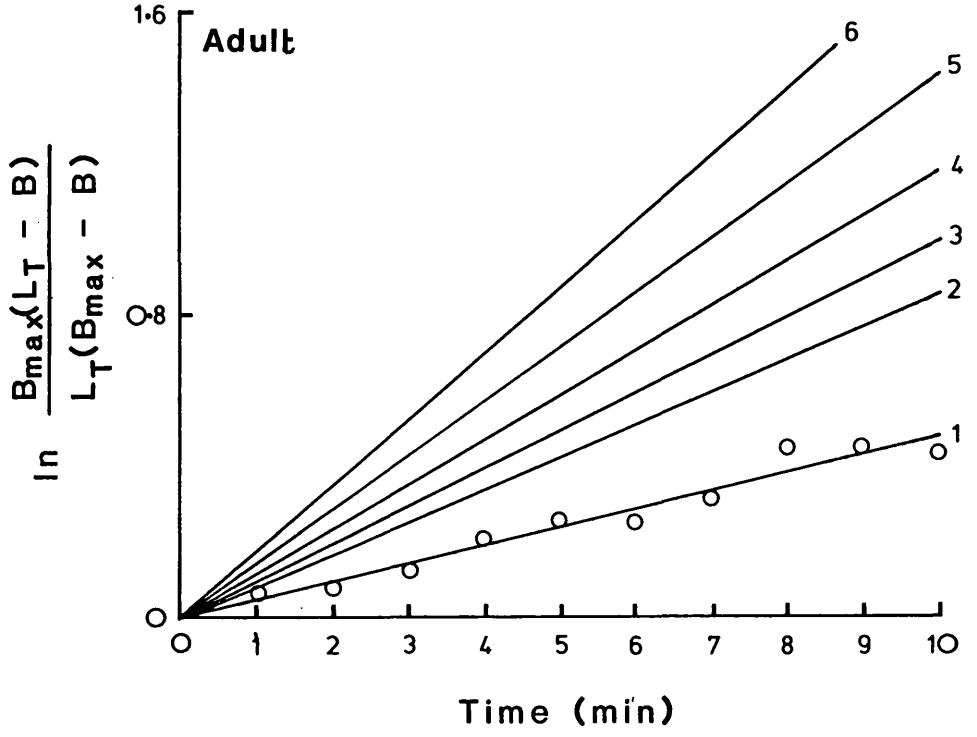
2. 9.50×10^5 $M^{-1}s^{-1}$

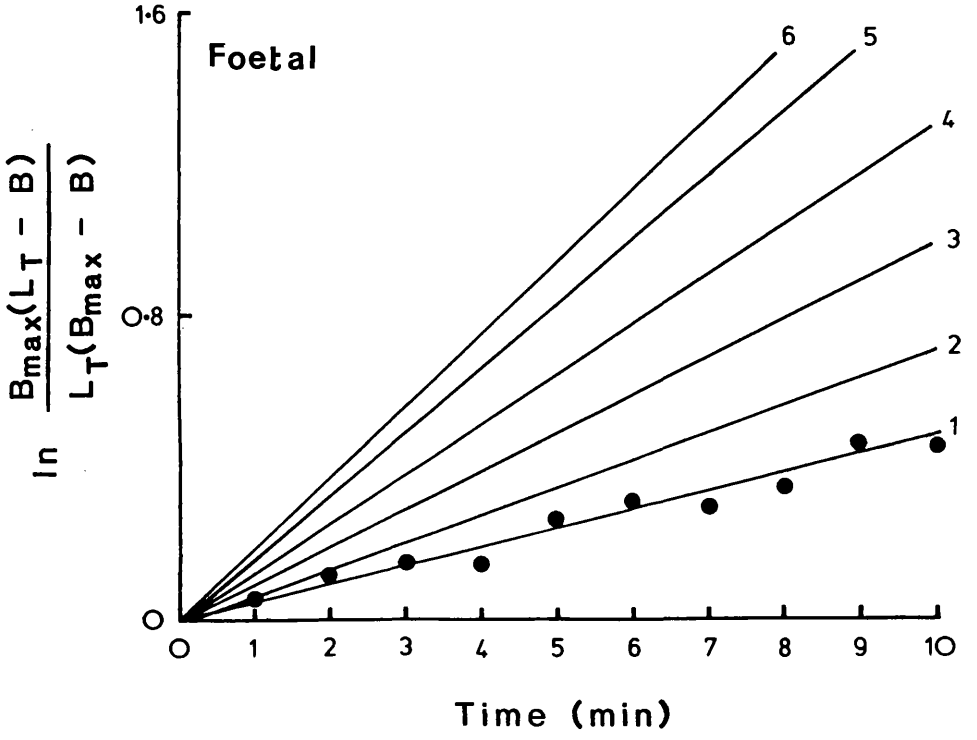
3. 8.47×10^5 $M^{-1}s^{-1}$

4. 10.78×10^5 $M^{-1}s^{-1}$

5. 6.61×10^5 $M^{-1}s^{-1}$

6. 7.60×10^5 $M^{-1}s^{-1}$


Adult
1·6
0·8
0
$\ln \frac{B_{max}(L_T - B)}{L_T(B_{max} - B)}$
6
5
4
3
2
1
0 1 2 3 4 5 6 7 8 9 10
Time (min)


Foetal
1·6
0·8
0
$\ln \frac{B_{max}(L_T - B)}{L_T(B_{max} - B)}$
6
5
4
3
2
1
0 1 2 3 4 5 6 7 8 9 10
Time (min)

$$\ln \frac{B}{B_e - B} = k_{ob}t \qquad \ldots\ldots.4$$

where:- $k_{ob} = k_{+1}' + k_{-1}$

and $k_{+1}' = k_{+1}L_T$.

So, if k_{ob} is determined at several ligand concentrations, allowing the derivation of a family of pseudo first order plots, then plotting k_{ob} against L_T will give a straight line with slope equal to k_{+1} and y-intercept equal to k_{-1}. Figure 44 shows the pseudo first order derivations from the initial progress curves shown in Figure 42, and Figure 45 the plots of k_{ob} against L_T obtained from these data. The rate constants so obtained are given in Table 16(b).

5.3.2 Dissociation rate constant (k_{-1})

The rate constant for dissociation of [^{125}I]α-BGT-AChR complex was determined in 2 ways:-

(a) From the relationship $k_{ob} = k_{+1}L_T + k_{-1}$

This is explained in the previous section and the results given in Table 17.

(b) Displacement of [^{125}I]α-BGT by unlabelled α-BGT

The equation:-

$$\frac{d(RL)}{dt} = k_{+1}[R][L] - k_{-1}[RL] \qquad \ldots\ldots.1$$

can be reduced to the equation

$$\frac{d(RL)}{dt} = -k_{-1}[RL] \qquad \ldots\ldots.5$$

if the rebinding of the radioactive ligand is prevented by, for

Figure 44. Derivation of association rate constants from pseudo first order treatment of binding data in Figure 42.

The slopes of the lines give values for k_{ob}.

For explanation see text.

Adult:- 1. $10.44 \times 10^{-4}\ s^{-1}$

2. $14.24 \times 10^{-4}\ s^{-1}$

3. $18.70 \times 10^{-4}\ s^{-1}$

4. $21.26 \times 10^{-4}\ s^{-1}$

5. $24.90 \times 10^{-4}\ s^{-1}$

6. $29.05 \times 10^{-4}\ s^{-1}$

Foetal:- 1. $10.23 \times 10^{-4}\ s^{-1}$

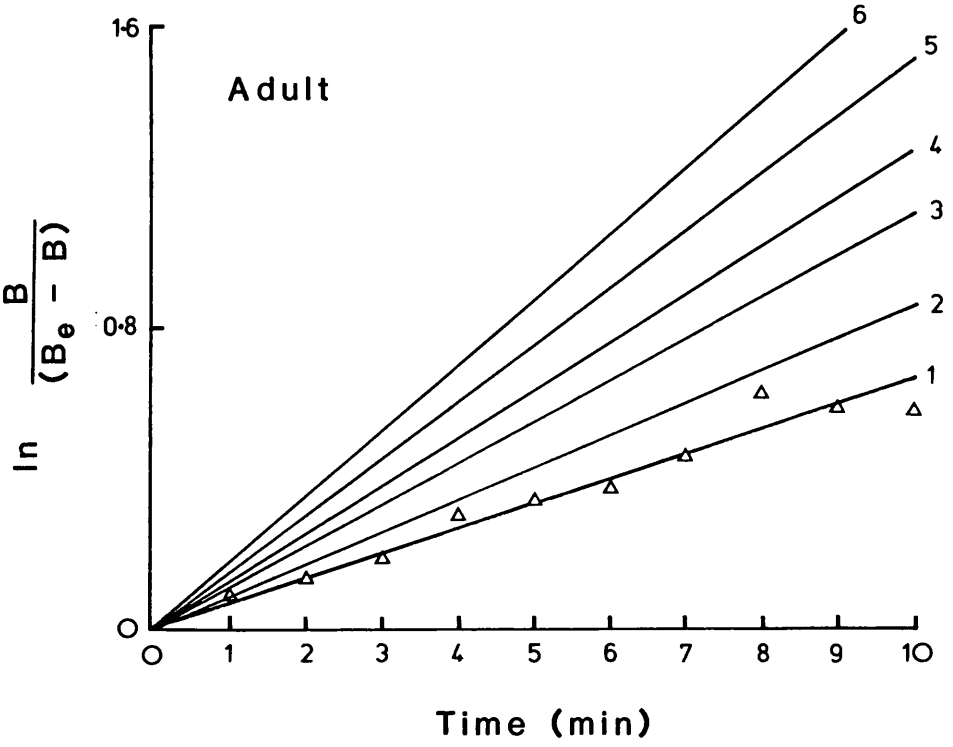
2. $13.01 \times 10^{-4}\ s^{-1}$

3. $17.67 \times 10^{-4}\ s^{-1}$

4. $32.60 \times 10^{-4}\ s^{-1}$

5. $29.13 \times 10^{-4}\ s^{-1}$

6. $31.67 \times 10^{-4}\ s^{-1}$


Adult
1·6
0·8
0
ln B/(Be − B)
0 1 2 3 4 5 6 7 8 9 10
Time (min)
1
2
3
4
5
6

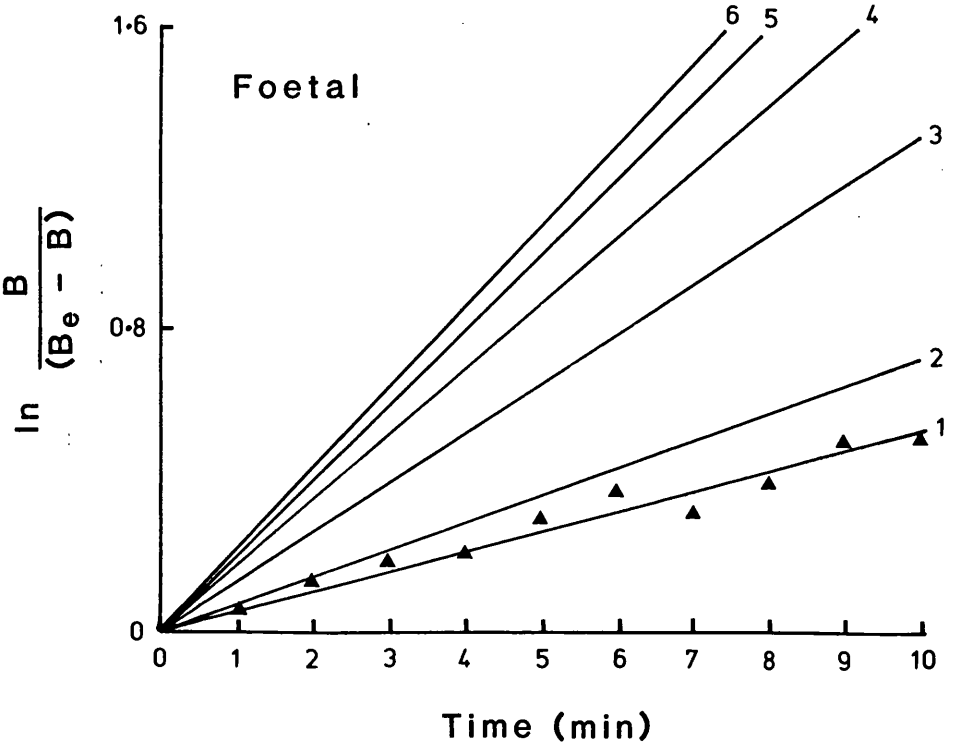

Foetal
1·6
0·8
0
ln B/(Be − B)
0 1 2 3 4 5 6 7 8 9 10
Time (min)
1
2
3
4
5
6

Figure 45. A plot of the k_{ob} values obtained from the previous graph against ligand concentration

The slope is equal to the assocation rate constant, k_{+1}; the y-intercept is equal to the dissociation rate constant, k_{-1}; and the -x-intercept is equal to the equilibrium constant, K_D, giving values of:-

Adult:- $k_{+1} = 5.52 \times 10^{5}\ M^{-1}\ s^{-1}$

$k_{-1} = 7.01 \times 10^{-4}\ s^{-1}$

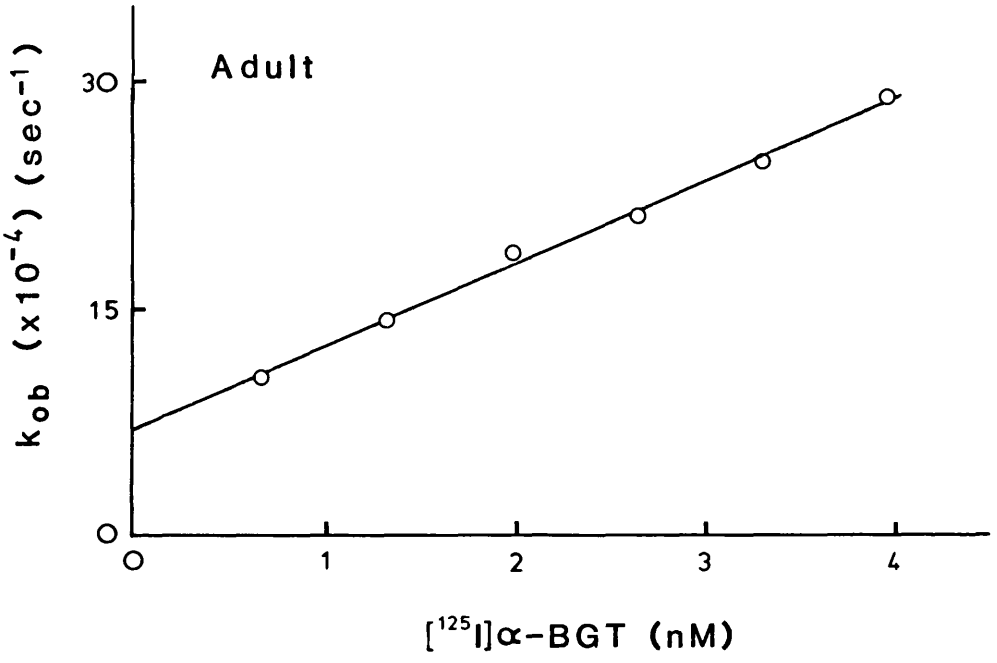
$K_D = 1.27 \times 10^{-9}\ M$

Foetal:- $k_{+1} = 6.94 \times 10^{5}\ M^{-1}\ s^{-1}$

$k_{-1} = 5.36 \times 10^{-4}\ s^{-1}$

$K_D = 0.77 \times 10^{-9}\ M$

The values obtained from these graphs are given in Table 16.


Adult
30
15
0
0
1
2
3
4
k_{ob} ($x10^{-4}$) (sec^{-1})
[^{125}I]α-BGT (nM)

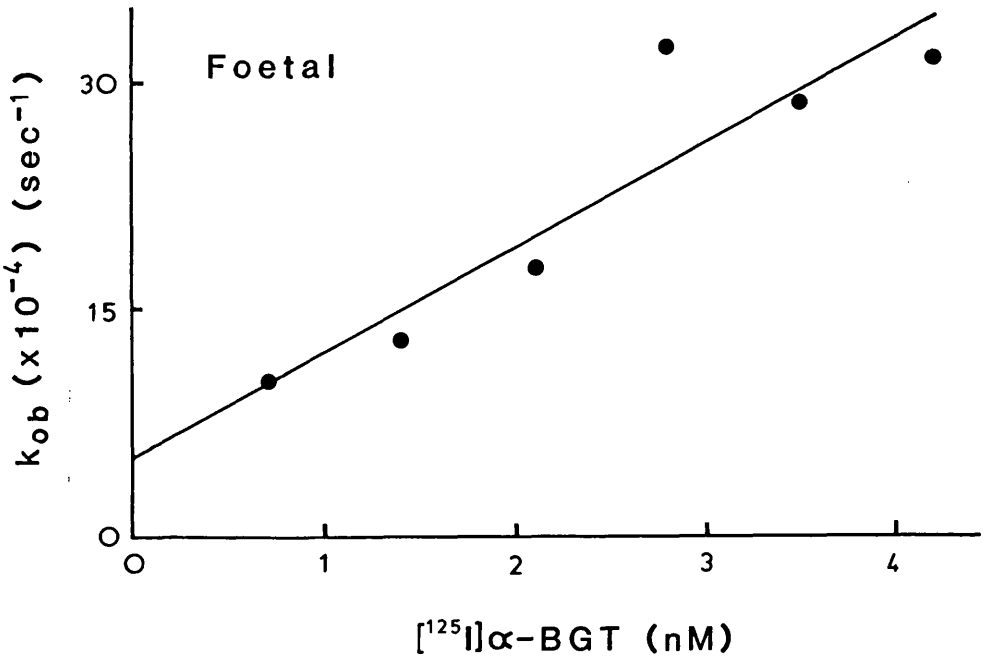

Foetal
30
15
0
0
1
2
3
4
k_{ob} ($x10^{-4}$) (sec^{-1})
[^{125}I]α-BGT (nM)

Table 16. Kinetic constants for association of [^{125}I]α-BGT to AChR

(a) Second order treatment of data

AChR type	k_{+1} ($\times 10^5 M^{-1} s^{-1}$)	SD	n
Adult 1	10.60	3.79	6
2	8.20	1.93	6
Foetal 1	12.41	3.63	6
2	9.71	3.10	6

(b) Pseudo first order treatment of data

AChR type	k_{+1} ($\times 10^5 M^{-1} s^{-1}$)	k_{-1} ($\times 10^{-4} s^{-1}$)	K_D (nM)
Adult 1	5.52	7.01	1.27
2	5.96	3.19	0.54
Foetal 1	10.27	2.54	0.25
2	6.94	5.36	0.77

Each value is the mean of 2 determinations

example, adding a large excess of non-radioactive ligand so that all free binding sites become occupied by the added unlabelled ligand. Integration of equation 5 gives:-

$$\ln \frac{B}{B_o} = -k_{-1}t \qquad \ldots\ldots.6$$

and plotting ln B/B_o against t gives a line with slope equal to $-k_{-1}$. The binding reaction between [^{125}I]α-BGT and AChR was allowed to proceed to equilibrium, after which, at an arbitrary time (t = 0), the rebinding of [^{125}I]α-BGT was prevented by the addition of a large excess of unlabelled α-BGT, and the amount bound was determined at various times thereafter. Representative time courses for dissociation of foetal AChR - [^{125}I]α-BGT and adult AChR - [^{125}I]α-BGT complexes are shown in Figure 46 and the resulting logarithmic plots in Figure 47. The slopes of the lines from the logarithmic plots give estimates of k_{-1}. The dissociation in both cases was seen to be biphasic, with a greater contribution by the faster initial phase which represented 60 - 80% loss of bound ligand in 4 - 6 hours. A summary of the dissociation rate constants so obtained is given in Table 17.

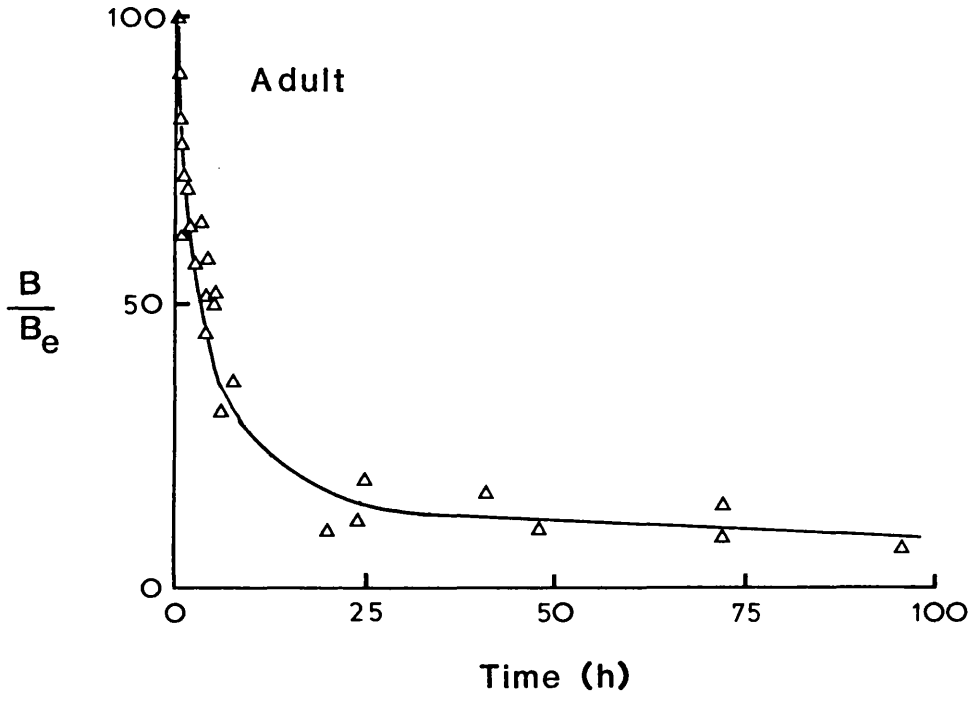
5.3.3 Equilibrium Dissociation Constant (K_D)

(a) From the equation $K_D = k_{-1}/k_{+1}$

See "Results" section 5.3.1 "Association Rate Constants" and summary of results in Table 18.

Figure 46. Dissociation of [^{125}I]α-BGT-AChR complexes measured after addition of excess unlabelled α-BGT at equilibrium

Samples of detergent extracts of human adult or foetal AChR were incubated with [^{125}I]α-BGT in the presence and absence of a large molar excess of unlabelled α-BGT. Excess unlabelled ligand (10^{-5} M) was added and the reaction stopped at various time intervals by the addition of cold toxin binding assay buffer and rapid filtration (see "Methods" section 7.2).


100
Adult
B/Be
50
0
0
25
50
75
100
Time (h)

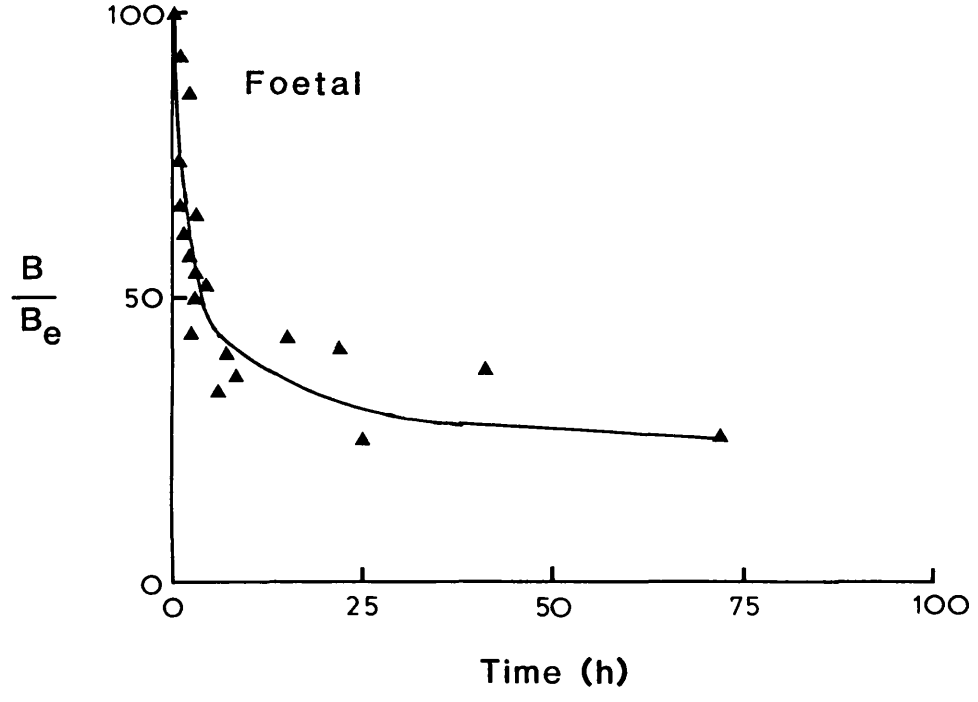

100
Foetal
B/Be
50
0
0
25
50
75
100
Time (h)

Figure 47. Logarithmic plots of the dissociation data in Figure 46.

The slopes of the lines give values of $-k_{-1}$, which are:-

Adult:- $0.58 \times 10^{-4}\ s^{-1}$

$7.50 \times 10^{-6}\ s^{-1}$

Foetal:- $0.70 \times 10^{-4}\ s^{-1}$

$5.85 \times 10^{-6}\ s^{-1}$

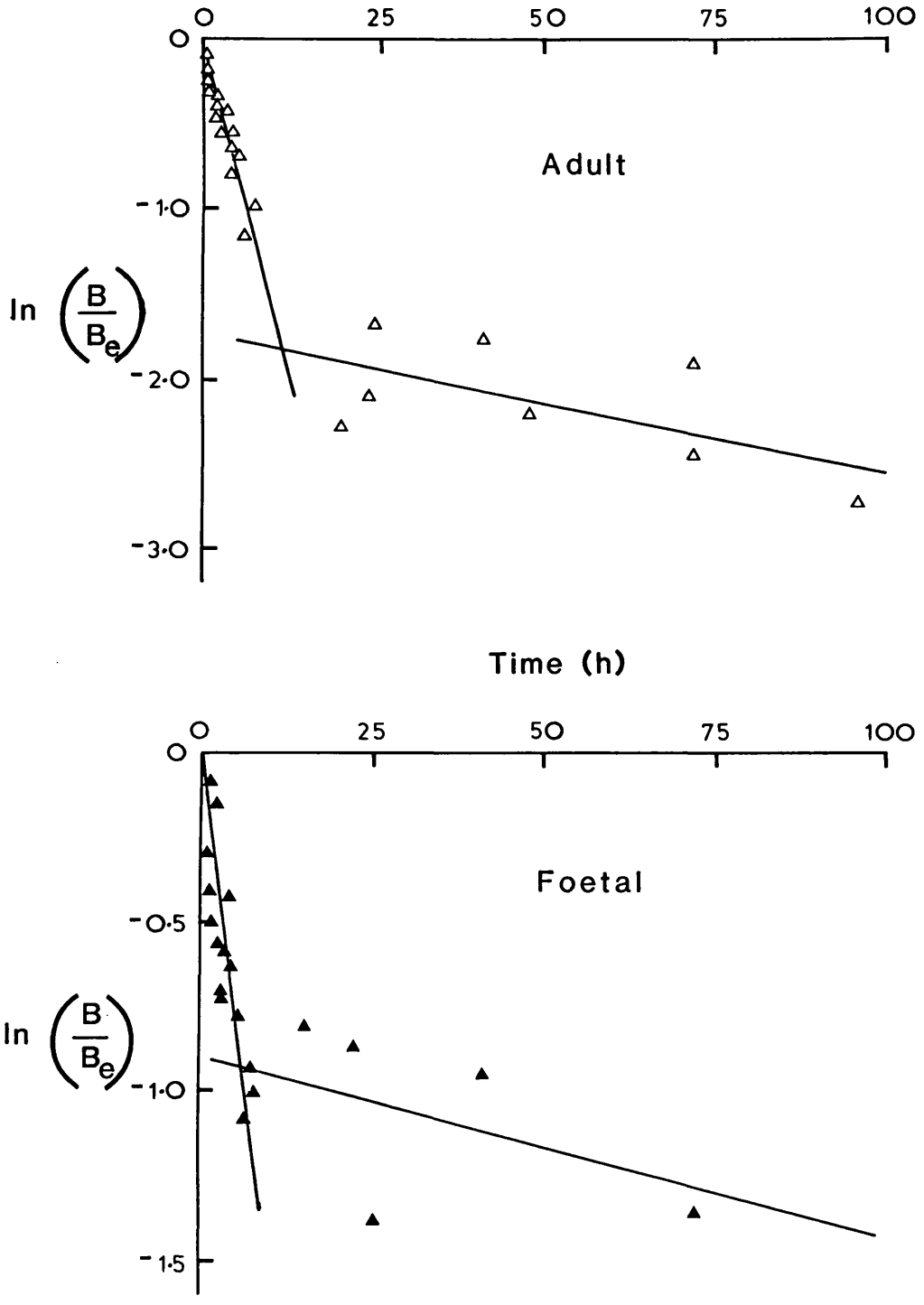

0
25
50
75
100
0
-1·0
-2·0
-3·0
Adult
ln (B/Be)
Time (h)
0
25
50
75
100
0
-0·5
-1·0
-1·5
Foetal
ln (B/Be)

Table 17. Kinetic constants for dissociation of [^{125}I]α-BGT labelled AChR complexes

AChR type		k_{-1} (fast) ($\times 10^{-4}$ s^{-1})	k_{+1} (slow) ($\times 10^{-6}$ s^{-1})
Adult	1	0.58	7.50
	2	0.73	12.80
Foetal	1	0.70	5.85
	2	0.74	9.10

Each value is the mean of 2 determinations

(b) Equilibrium binding of [^{125}I]α-BGT

[^{125}I]α-BGT binding to detergent-solubilized foetal and adult AChR was studied over a range of [^{125}I]α-BGT concentrations, and representative saturation curves are shown in Figure 48. Non-specific binding, which was measured in the presence of excess unlabelled α-BGT, was linear and accounted for 25 - 85% of the total binding. The specific binding was saturable, describing a rectangular hyperbola which can be reduced to a straight line form by the Scatchard equation (Scatchard, 1949):-

$$\frac{B}{F} = \frac{-B}{K_D} + \frac{B_{max}}{K_D} \qquad .....7$$

where:- B = concentration of binding sites

B_{max} = maximum concentration of binding sites (ie. at equilibrium)

F = concentration of free sites

K_D = equilibrium binding constant

The K_D is the negative reciprocal of the slope of the line and B_{max} is the intercept on the abscissa. Figure 49 shows such straight line plots obtained from the data in Figure 48 which indicate a single class of binding sites. The B_{max} obtained from Scatchard analysis agreed well with values obtained in ammonium sulphate assays. The results were also subjected to analysis by Hill plots (Hill, 1910):-

$$\log_{10} \frac{B}{B_{max} - B} = n \log_{10} F - \log_{10} K_D' \qquad .....8$$

Figure 48. Equilibrium binding of [^{125}I]α-BGT to detergent solubilized human adult AChR

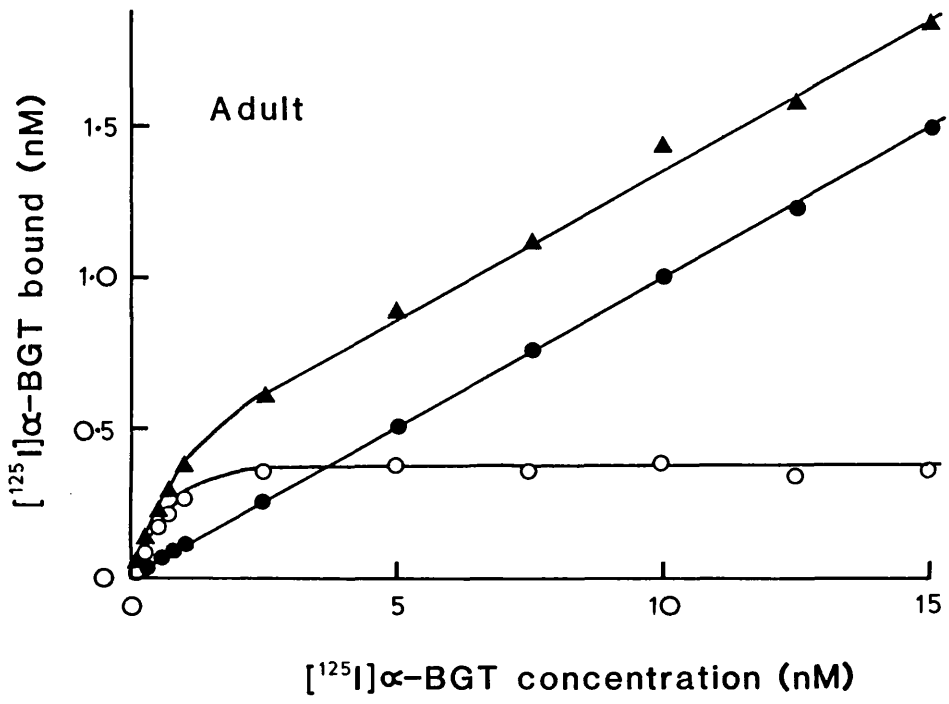
Samples of detergent extracts of adult and foetal AChR were incubated with increasing concentrations of [^{125}I]α-BGT in the presence and absence of a large molar excess of unlabelled α-BGT, followed by precipitation with ammonium sulphate. Bound radioactivity was recovered by filtration on glass fibre filter discs (see "Methods" section 7.1).

▲ Total [^{125}I]α-BGT bound

● Non-specific binding

o Specific binding

Binding data from a representative experiment. Points are means of triplicate determinations.


Adult
[125I]α-BGT bound (nM)
1·5
1·0
0·5
0
0
5
10
15
[125I]α-BGT concentration (nM)

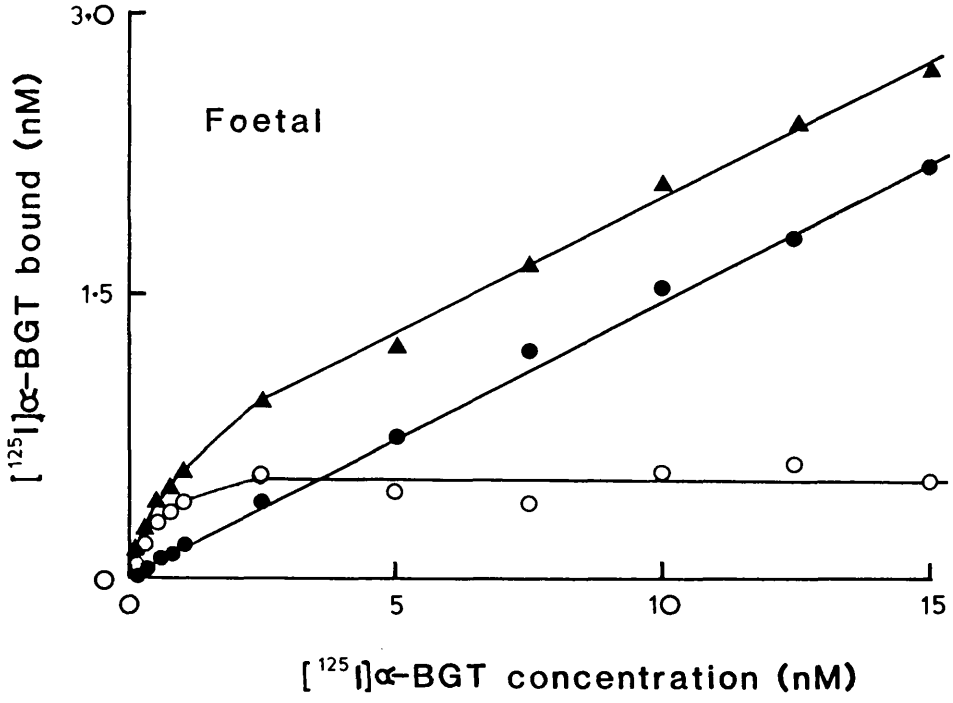

Foetal
[125I]α-BGT bound (nM)
3·0
1·5
0
0
5
10
15
[125I]α-BGT concentration (nM)

Figure 49. Scatchard analysis and Hill plots of equlibrium binding data from Figure 48.

Scatchard analysis

Adult:- K_D(app) = 0.31 nM

B_{max} = 0.80 pmoles/ml [^{125}I]α-BGT binding sites

r^2 = 0.99

Foetal:- K_D(app) = 0.10 nM

B_{max} = 0.45 pmoles/ml [^{125}I]α-BGT binding sites

r^2 = 0.99

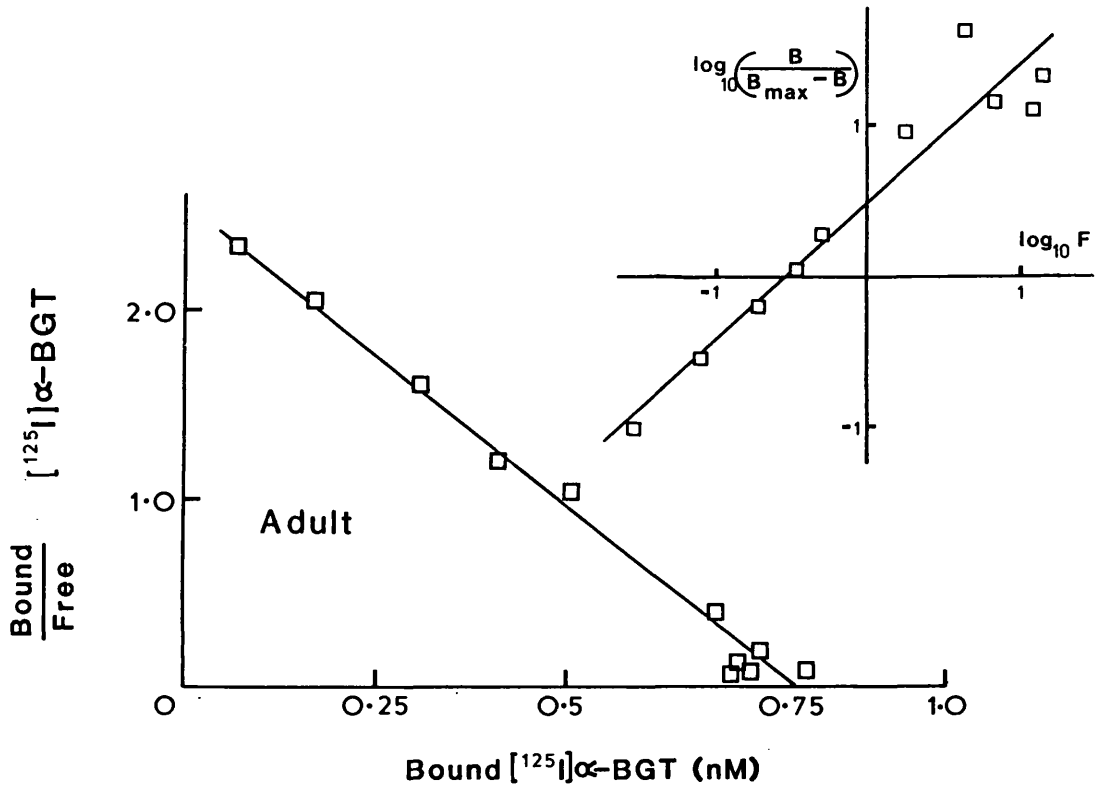
Hill plots

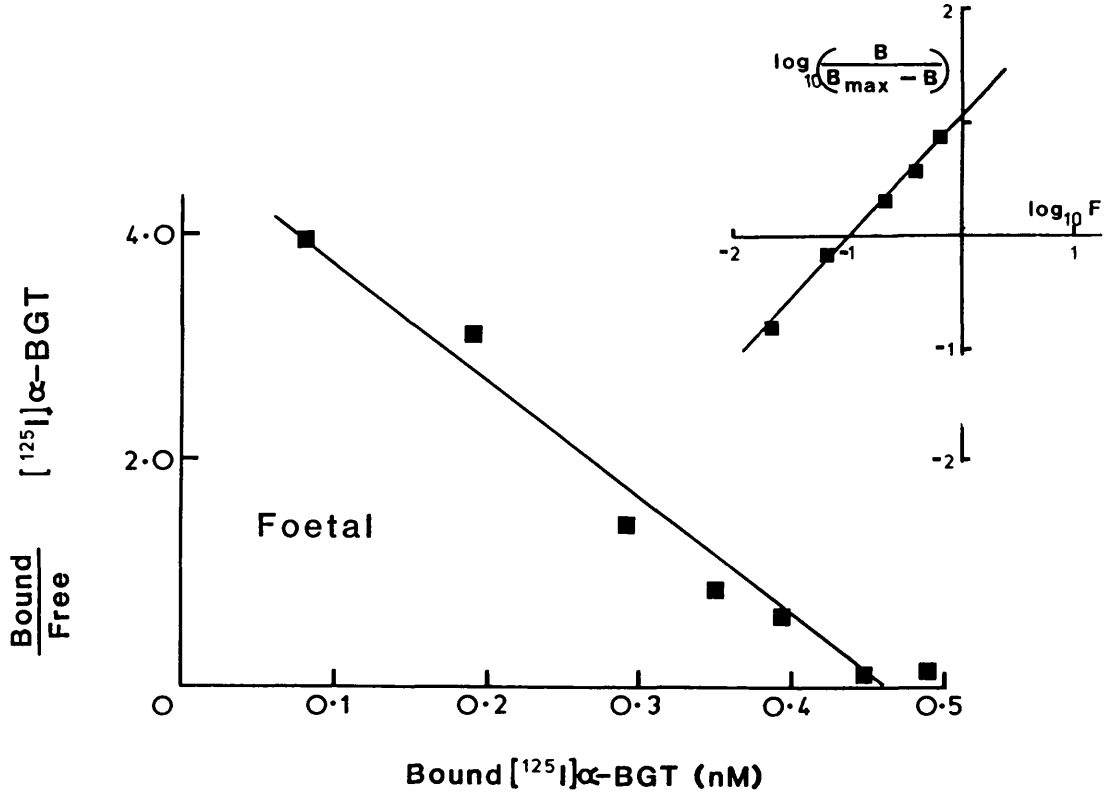
Adult:- N_H = 0.90

r^2 = 0.96

Foetal:- N_H = 0.99

r^2 = 0.99


Bound / Free [125I]α-BGT
2·0
1·0
Adult
0
0·25
0·5
0·75
1·0
Bound [125I]α-BGT (nM)
log10 (B / Bmax − B)
log10 F
1
-1
1
-1


Bound / Free [125I]α-BGT
4·0
2·0
Foetal
0
0·1
0·2
0·3
0·4
0·5
Bound [125I]α-BGT (nM)
log10 (B / Bmax − B)
log10 F
2
-2
-1
1
-1
-2

where:- B = concentration of [^{125}I]α-BGT bound

B_{max} = maximum number of binding sites (determined from intercept on the y-axis in the Scatchard plot)

F = concentration of [^{125}I]α-BGT free

K_D' = a composite constant composed of the intrinsic dissociation constant, K_D and interaction factors that determine the degree to which K_D is altered at each discrete binding step

n = N_H = Hill coefficient

The plot quantitates the deviation of a receptor-ligand interaction from the classic mass action hyperbola, the slope of the line giving the Hill coefficient. Figure 49 shows the plots obtained from the data given in Figure 48, from which it can be seen that straight lines were obtained, with slopes of 1.01 and 0.86 for adult AChRs and 0.93 and 0.94 for foetal AChRs, which indicates a single class of binding sites. Table 18 gives a summary of the apparent K_D values and Hill coefficients obtained.

In summary, the results show no difference in the interaction of AChR from human foetal or adult muscle with [^{125}I]α-BGT. Both receptor types showed similar association and dissociation kinetics, and the dissociation displays biphasic characteristics. Scatchard and Hill plots indicate a single class of binding sites. The observed equilibrium binding constants from the analysis are higher than those obtained from the ratio of rate constants, because the AChR concentration used was higher than the K_D in order to achieve sensitivity in the assay (Cuatrecasas & Hollenberg, 1976).

Table 18. Equilibrium binding constants and Hill coefficients for binding of [^{125}I]α-BGT to detergent extracts of AChR

AChR type		K_D ($\times 10^{-9}$ M)	SD	N_H	SD
Adult	1	2.35	0.79	1.01	0.03
	2	0.35	0.11	0.86	0.20
Foetal	1	1.06	0.71	0.93	0.14
	2	0.28	0.13	0.94	0.07

Each value is the mean of at least 3 determinations.

6. Interaction of human adult and foetal AChRs with anti-(AChR) antibodies in myasthenic serum and IgG

The nature of the antigenicity of the foetal AChR was compared with that of the adult AChR.

6.1 Relationship between antigenicity of foetal AChR and foetal age

Increasing concentrations of [^{125}I]α-BGT - labelled foetal AChR from individual foetuses were incubated with a fixed amount of myasthenic serum (patient 10), producing a saturation curve from which the anti-(AChR) antibody titre was determined. Figure 50(a) shows the plot of anti-(AChR) antibody titre against foetal age, from which it can be seen that the antibody titre measured stays fairly constant with foetal age and agrees well with values obtained by using adult muscle AChR. The assay was also performed "in reverse", where a large molar excess of the same myasthenic serum was incubated with a constant limited amount of [^{125}I]α-BGT - labelled AChR to give the percentage precipitation of the labelled complex. From Figure 50(b) it can be seen that the percentage precipitation of labelled AChR remained constant with increasing foetal age.

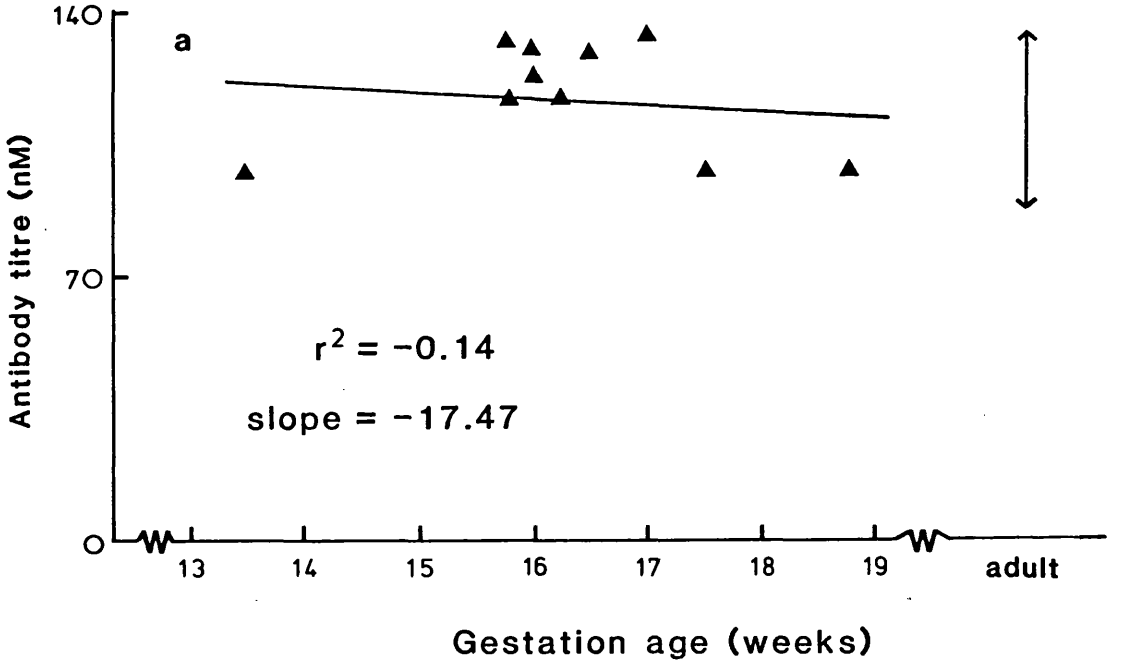
Since there did not appear to be any significant difference in the interaction of anti-(AChR) antibody with AChR of increasing foetal age, different aged foetuses were combined together to provide sufficient muscle to attempt purification of AChR and comparison with purified adult AChR.

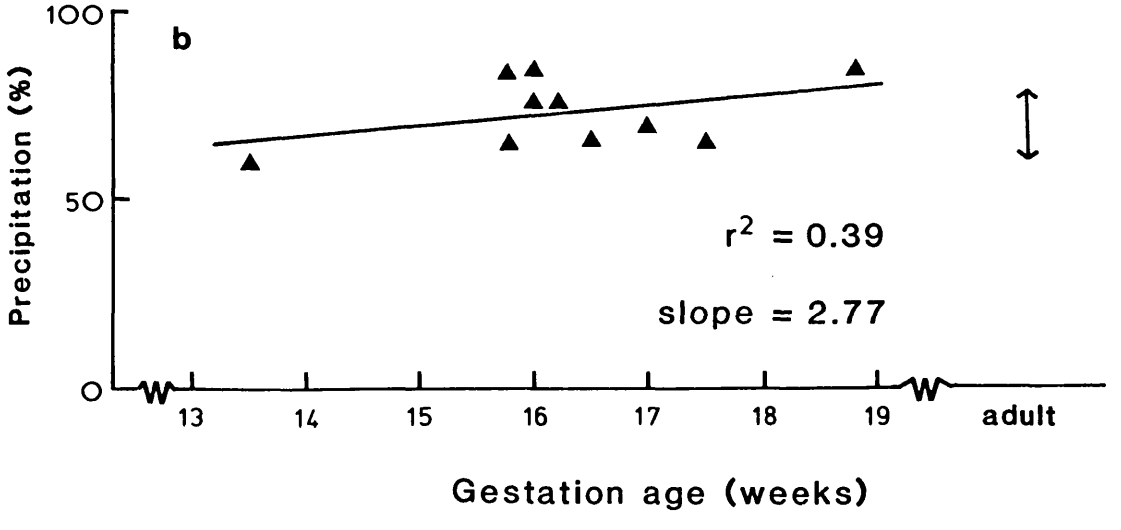
Figure 50. Relationship between antigenicity of foetal AChR and foetal age

Duplicate samples of [^{125}I]α-BGT labelled AChR (100 µl, labelled in the presence and absence of benzoquinonium chloride), were incubated with a fixed volume of myasthenic serum (from patient 10), followed by precipitation with goat anti-(human IgG) antiserum and recovery by centrifugation (see "Methods" section 8). Each point represents the mean of 3 separate determinations.

(a) Relationship between antibody titre and foetal age. [^{125}I]α-BGT labelled AChR (0.1 - 1.0 nM) was incubated with a fixed concentration of myasthenic serum (5 µl), followed by precipitation of the immune complex as described above.

(b) Relationship between percentage precipitation of [^{125}I]α-BGT labelled AChR by excess myasthenic serum and foetal age. [^{125}I] α-BGT labelled AChR (0.15 nM) was incubated with a large molar excess (10 µl) of serum, followed by precipitation of the immune complex as described above.


a
140
70
0
Antibody titre (nM)
$r^2 = -0.14$
slope = −17.47
13
14
15
16
17
18
19
adult
Gestation age (weeks)


b
100
50
0
Precipitation (%)
$r^2 = 0.39$
slope = 2.77
13
14
15
16
17
18
19
adult
Gestation age (weeks)

6.2 A comparison of the interaction of adult and foetal human AChRs with myasthenic sera and IgG

Serum and IgG from each of 3 myasthenic patients were assayed for anti-(AChR) antibodies by using 'crude' and purified preparations of foetal and adult human AChR in the radioimmunoassay (see "Methods" section 8). In each case, increasing concentrations of antigen were tested, producing a saturation curve from which antibody titre and affinity were determined. Representative saturation curves for the binding of affinity purified adult and foetal human AChR to anti-(AChR) antibodies from one myasthenic serum and corresponding IgG are shown in Figures 51 - 53. The V_{max} and apparent K_d values from the saturation curves from this and 2 other patients are given in Table 19. The curves were fitted by the Maximum Likelihood Method (Silvey, 1975), which, since normal errors of y-values were assumed, is the same as the least squares method for linear regression, ie the sum of squared distances from the plotted points to the fitted curve are minimized. The concept of curve fitting is straightforward, but the nature of the curve makes it necessary to perform the minimization iteratively. In order to do this a computer standard minimization package was used, and the curve fitted was of the form:-

$$y = \alpha \frac{\gamma^x - 1}{\gamma^x + \beta} \qquad \text{where:-}$$

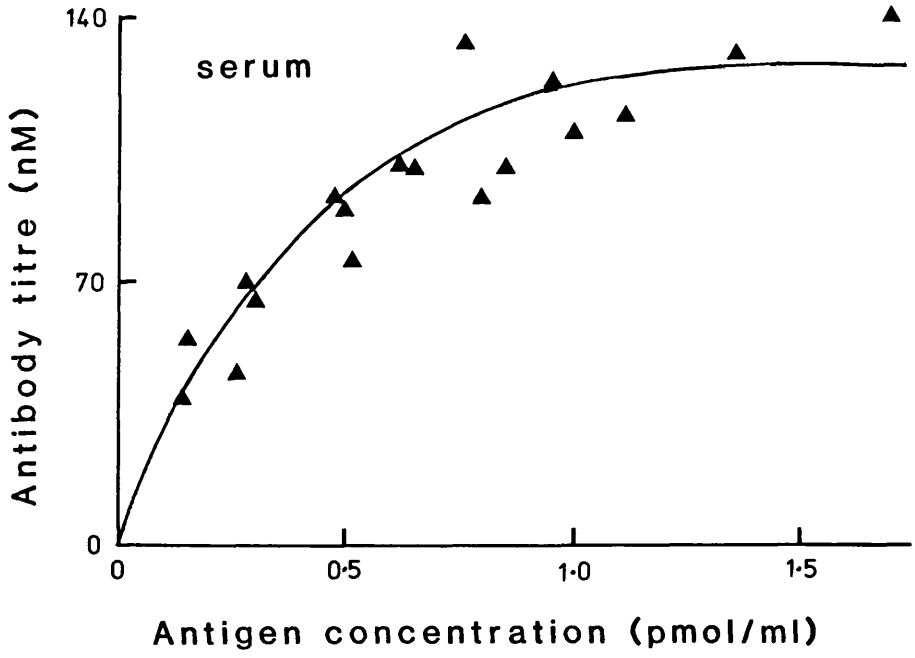
$\alpha = Y_{max}$

γ = a measure of how quickly y approaches Y_{max} as x increases

$\frac{\log (2+\beta)}{\log \gamma}$ is the x-value at half maximal saturation.

Figure 51. Saturation curves for the binding of purified adult (SVD) AChR preparations with anti-(AChR) antibodies in myasthenic serum and IgG from Patient 10.

An appropriate dilution of serum or IgG was assayed with increasing concentrations of antigen (see "Methods" section 8). Each point is the mean of duplicate determinations. The values are taken from 3 independent assays using 3 different adult (SVD) AChR preparations.


140
70
0
serum
Antibody titre (nM)
0
0·5
1·0
1·5
Antigen concentration (pmol/ml)

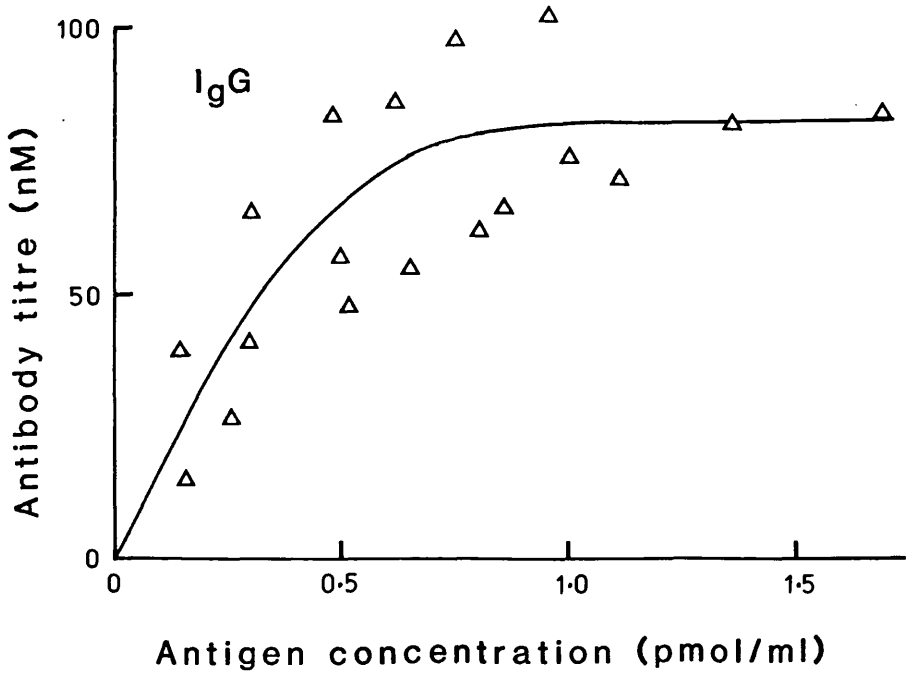
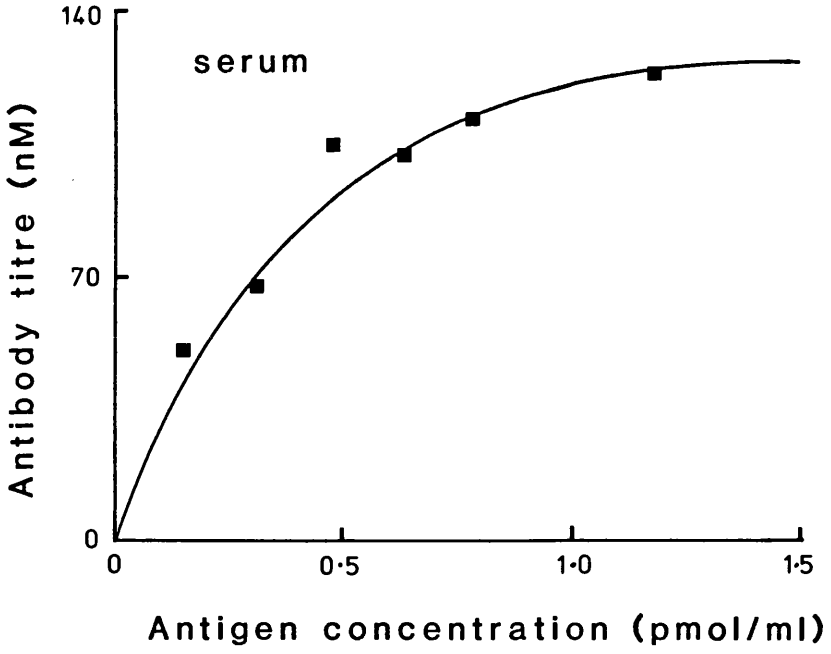

100
50
0
IgG
Antibody titre (nM)
0
0·5
1·0
1·5
Antigen concentration (pmol/ml)

Figure 52. Saturation curves for the binding of purified adult (RTA) AChR preparations with anti-(AChR) antibodies in myasthenic serum and IgG from Patient 10.

An appropriate dilution of serum or IgG was assayed with increasing concentrations of antigen (see "Methods" section 8). Each point is the mean of duplicate determinations. The values are taken from 1 assay using 1 adult (RTA) AChR preparation.


140
70
0
serum
Antibody titre (nM)
0
0·5
1·0
1·5
Antigen concentration (pmol/ml)

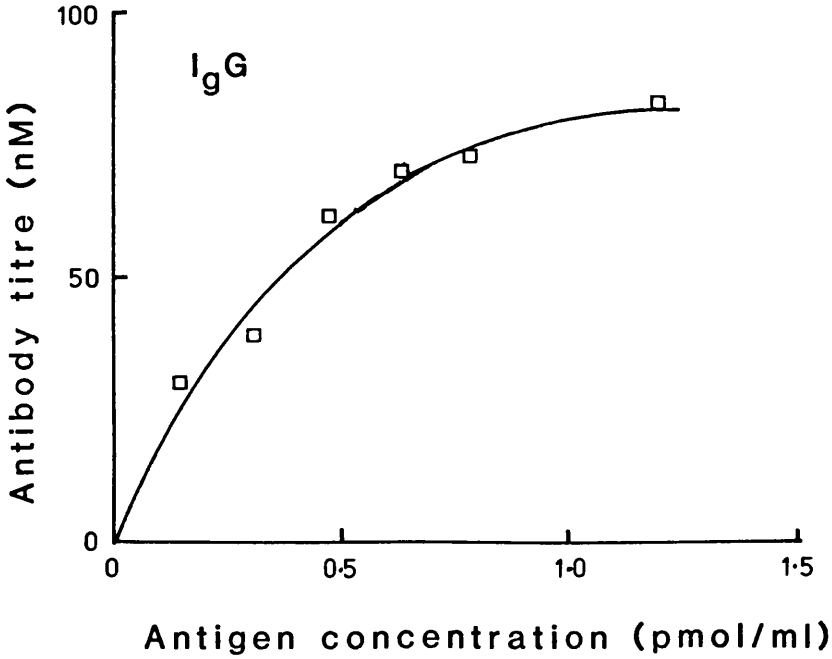
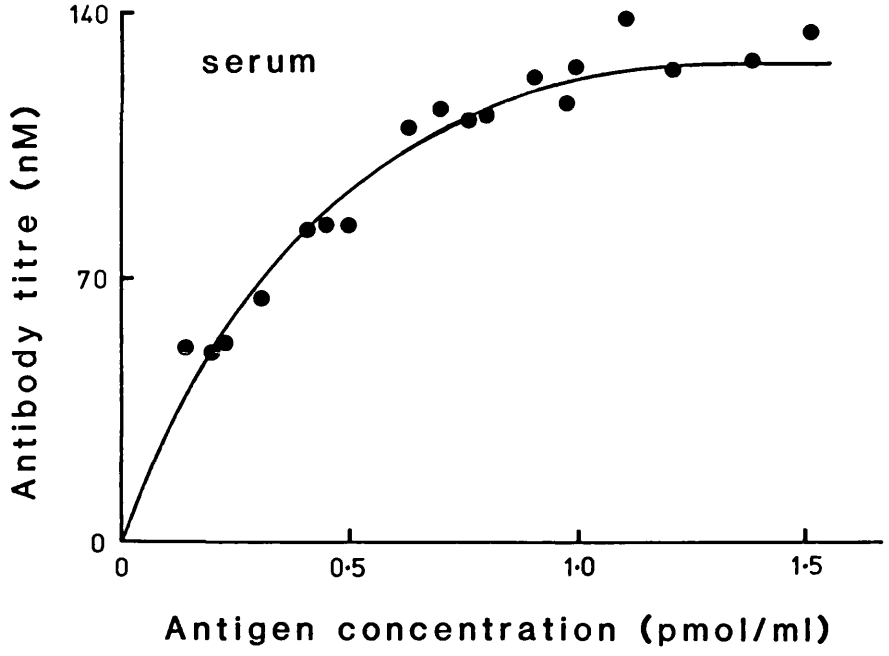

100
50
0
IgG
Antibody titre (nM)
0
0·5
1·0
1·5
Antigen concentration (pmol/ml)

Figure 53. Saturation curves for the binding of purified foetal AChR preparations with anti-(AChR) antibodies in myasthenic serum and IgG from Patient 10.

An appropriate dilution of serum or IgG was assayed with increasing concentrations of antigen (see "Methods" section 8). Each point is the mean of duplicate determinations. The values are taken from 2 independent assays using 2 different foetal AChR preparations.


140
70
0
serum
Antibody titre (nM)
0
0·5
1·0
1·5
Antigen concentration (pmol/ml)

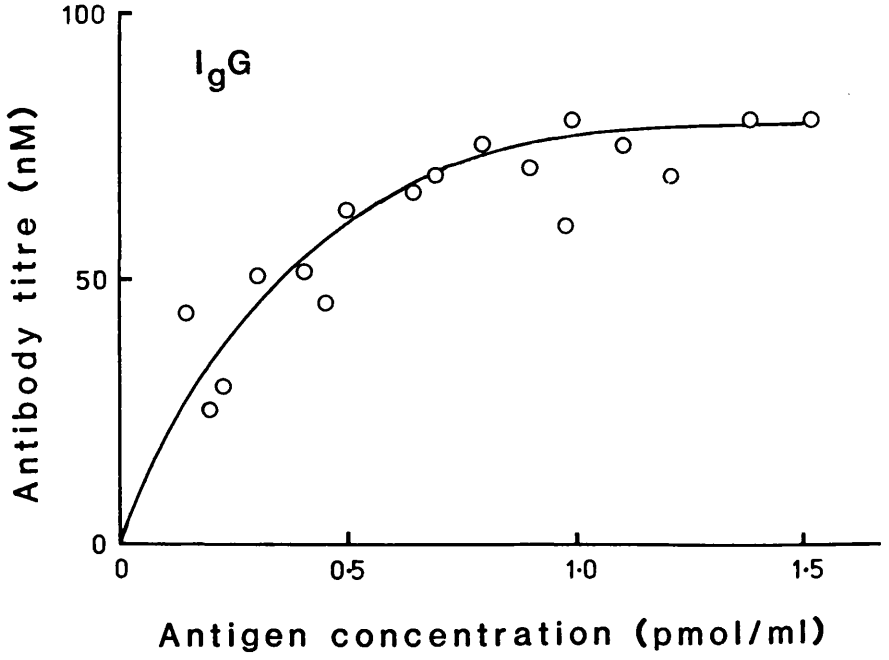

100
50
0
IgG
Antibody titre (nM)
0
0·5
1·0
1·5
Antigen concentration (pmol/ml)

Table 19. Comparison of binding data for myasthenic sera and IgG with different human AChR preparations

Data are taken from: 3 independent series of assays using 2 different foetal AChR preparations; 3 independent series of assays using 3 different adult SVD (severe vascular disease) preparations; 1 series of assays using 1 adult RTA (road traffic accident) AChR preparation. K_d values are referred to as app. K_d (apparent K_d) in that they represent average values for each sample of polyclonal antisera tested.

* Number of points on binding curve.

\+ Anti-(AChR) antibody titre x 10^{-10} M.

X^2 is a measure of the similarity of pairs of binding curves (see "Results" section 6.2). Significant difference at the 95% level between the two curves requires that $X^2 > 7.8$.

Antigen	Patient 14				Patient 11							
	Serum				Serum				IgG			
	n*	Titre	K_d (nM)	X^2	n	Titre	K_d (nM)	X^2	n	Titre	K_d (nM)	X^2
Foetal AChR	18	122	0.30	1.30	18	210	0.34	4.05	18	149	0.39	3.10
Adult (SVD) AChR	12	124	0.30		18	192	0.31		18	128	0.32	
Adult (RTA) AChR												

Antigen	Patient 10							
	Serum				IgG			
	n	Titre	K_d (nM)	X^2	n	Titre	K_d (nM)	X^2
Foetal AChR	18	1416	0.32	3.57	17	815	0.25	1.99
Adult (SVD) AChR	16	1383	0.31		18	828	0.25	0.17
Adult (RTA) AChR					6	837	0.26	

The curve is of similar form to the Michaelis-Menten equation which can be expressed as:-

$$y = \frac{\alpha x}{\beta + x}$$

and gives a rectangular hyperbola. The introduction of the term γ into this equation enables the curve to flatten out at lower values of x than is otherwise possible. Straight line derivations from the saturation curves had been tried, but were discounted since they did not fit well due to the bias obtained when double- or half-reciprocal plots were fitted. Even the use of weighted linear regression failed to give estimates of K_m and V_{max} which were consistent with values obtained by "eye" directly from the original saturation curves. Standard errors for the parameter estimates were computed from the estimated values of $\alpha, \beta, \gamma, \sigma^2$ (the estimated variance) and the x- and y- values. The usual approximate 95% confidence interval is then $p \pm 2 \times S.E.(p)$, where p is the parameter estimate. The Maximum Likelihood Theory makes it possible to construct an asymptotic likelihood ratio test which can be used to determine whether the same curve can be fitted to 2 data sets. In order to determine whether V_{max} and the apparent K_d for adult and foetal antigens are the same the following null hypothesis was tested:-

H_o : the two curves, both of the form:-

$$y = \alpha \frac{\gamma^x - 1}{\gamma^x + \beta}$$

are the same, ie the α, β, γ parameters are the same for both curves

against the alternative hypothesis:-

H_1 : the two curves are different, ie they have differing α, β, γ parameters.

The statistic X^2 was looked at, where:-

$$X^2 = (\text{size of pooled sample}) \times \log \frac{\text{RSS for pooled curve}}{\text{RSS curve 1 + RSS curve 2}}$$

RSS = residual sum of squares

= sum of squares of distances of points from the fitted curve.

If the two curves are the same, then X^2 will have an approximately χ^2 (chi-squared) distribution with 3 degrees of freedom; whereas if the curves are different, the value of X^2 will be larger. From χ^2 tables, using 3 degrees of freedom, it can be seen that the value of X^2 needs to exceed 7.8 to be significant at the 95% level, ie a value of X^2 greater than 7.8 would mean that the curves were significantly different. X^2 values for the pairs of saturation curves obtained from purified preparations are given in Table 19. Parameters obtained from the various saturation curves are shown in Table 19, in which it can be seen that the values of anti-(AChR) antibody titre and apparent K_d for a given serum or IgG sample are very similar irrespective of whether purified adult AChR or purified foetal AChR is used as the antigen. This is reflected in the X^2 values obtained from pairs of fitted curves, which in no case exceeds 4.10. Crude extracts, in which receptor concentrations were frequently below 0.5 nM, were less suitable for these purposes, since concentration of the antigen, necessary in order to obtain complete saturation, led to a greater scatter of points and incomplete binding curves. The validity of using amputated muscle from patients with diabetic gangrene or severe vascular disease as a

source of normal junctional AChR in the assay was checked by using AChR purified from normal adult muscle amputated following a road traffic accident to assay serum and IgG from patient 10. No significant difference was found between the fitted saturation curves so obtained and those obtained with AChR from ischaemic muscle (Figure 52, Table 19).

DISCUSSION

For many years the neuromuscular junction has been recognized as the general region of the defect in the human disease myasthenia gravis (Walker, 1934). This complex neurological syndrome is the result of an autoimmune response directed against the nicotinic AChR at the post-synaptic membrane, where the impairment of the function of the receptor characteristically produces flaccid paralysis and susceptibility to fatigue of skeletal muscle.

The physiological function of the AChR at the neuromuscular junction is to interact with the neurotransmitter acetylcholine (ACh), thus producing a change in permeability of the post-synaptic membrane, which eventually leads to muscle contraction. Direct measurement of AChR function in its membrane environment by electrophysiological methods has been complemented by the more recent advances in biochemical techniques which, facilitated by the unique ligand α-BGT and a rich source of AChR, namely Torpedo electroplaques, have enabled the isolation and characterization of the AChR - this has necessitated the development of appropriate biochemical assays. The most widely used method for assay of isolated AChRs relies on the fact that the binding site for the natural transmitter is a highly specific one. However, in practice, ACh is not the preferred ligand for use, as the interaction of ACh with its receptor is readily reversible under experimental conditions ($K_D \approx 10^{-5}$M), which is to be expected if the neurotransmitter is not to cause pharmacological blockade; and also, "crude" preparations of AChR contain high levels of endogenous AChE which leads to rapid hydrolysis of ACh (Eldefrawi et al., 1972). The

requirements for a highly specific, high affinity, non-degradable ligand are met by α-BGT, which can be radiolabelled to high specific activity without serious loss of biological activity. Additionally, the receptor-ligand complex can be separated from free ligand on the basis of MW, charge difference and immunoreactivity and thus provides a highly versatile tool for identification and quantitation of AChR. Furthermore, by using the toxin in conjunction with human AChR, it has been possible to develop sensitive methods for the detection and determination of anti-(AChR) antibodies in sera from MG patients (Lindstrom et al., 1976a).

[^{125}I]α-BGT as a probe for n-AChR

Ligands for binding assays are commonly radiolabelled with tritium, or, in the case of proteins, with iodine. Because tritium replaces H atoms usually present in the ligand, tritiation produces labelled ligands which are biologically indistinguishable from their native counterparts. A further advantage stems from the long radiochemical half life of tritium (12.26 years) which, coupled with its low radiation energy (0.0186 Mev), allows extended storage without the need for frequent preparation or re-purification. However, specific radioactivities obtained from the introduction of [^{3}H] into a molecule are much lower than can be achieved by radioiodination eg 1 atom of [^{131}I] provides the same number of disintegrations per unit time as 600 atoms of [^{3}H]. [^{131}I] is unattractive because of its short half life (8 days), compared with [^{125}I] (60 days) and it is therefore [^{125}I] which is usually the I isotope used for radiolabelling. A disadvantage of using radioiodinated proteins is the radiation-induced destruction which

frequently alters biological activities of proteins and produces variable mixtures of labelled products with different properties. However, this disadvantage is often offset by the high relative specific radioactivities achieved, which have proved invaluable in detecting small quantities of biological materials.

A number of tritiation methods have been applied to neurotoxins (eg α-BGT) that specifically react with n-AChRs. Mono [^{3}H]acetyl-α-BGT (Barnard et al., 1971) has the same affinity for muscle receptors as native toxin (Barnard et al., 1975), but has specific radioactivities too low for sensitive analyses of most cellular receptors. N-[propionyl-^{3}H] propionylated α-BGT (Dolly et al., 1981) on the other hand, has a specific radioactivity of 46 Ci/mmol, is 90 - 100% biologically active and has been shown to be as sensitive as [^{125}I]α-BGT in the determination of AChR and anti-(AChR) antibodies. In view of the cumbersome counting procedures associated with [^{3}H], iodination was selected for routine use and gave consistently labelled protein with high specific radioactivities (see "Results" section 1.1). When the mono- and di-iodinated derivatives of α-BGT were compared, with [^{3}H]α-BGT, in their reaction with n-AChR in binding assays, [^{3}H]α-BGT, unfractionated [^{125}I]α-BGT and mono-iodinated α-BGT gave similar results, validating this choice. The binding of di-iodinated α-BGT was consistently lower (see "Results" section 1.6), an observation also reported by Vogel et al. (1972) and attributed to a partial unfolding of the toxin molecule leading to a reduced affinity towards toxin binding sites (Lucasiewicz et al., 1978).

The values obtained here for biological activities of the different labelled toxins depended on the method of separation of

bound and free species - values obtained by gel filtration being consistently higher than those obtained from filtration on DEAE-cellulose filter discs. One possible explanation for this could be that iodinated α-BGT might aggregate, and while this would still pass through DEAE-cellulose filters as a function of its charge, aggregates may co-chromatograph with [^{125}I]α-BGT-AChR upon gel filtration and give the apparent increase in biological activity observed (Krohn et al., 1972; Sherman et al., 1974; Jailkhani et al., 1984).

Detergent extraction and partial purification of AChR

AChRs from several species were solubilized using the non-ionic detergent Triton X-100. Some detergent extracts of human adult and foetal AChR were purified by affinity chromatography on α-toxin coupled Sepharose-4B followed by biospecific elution with carbachol and concentration on DEAE-cellulose.

Yields of AChR obtained from mammalian muscle are consistently lower than those obtained for Torpedo (see Table 20), which necessitates the use of a bulk source of tissue. Post mortem muscle is one such source, but the low yields of AChR obtained from such tissue (0.1 pmol [^{125}I]α-BGT binding sites/g muscle) makes it an unsatisfactory starting material. Denervation of muscle, which is followed by proliferation of AChR (see "Introduction" p. 10) is one method which has been used in experimental animals to increase the yield of AChR; however, surgical denervation of human adult muscle is clearly not feasible. Amputated limbs from diseased patients are a readily available source of muscle, and have the added advantage that the muscle can be quickly dissected and frozen, so reducing the

liklihood of proteolytic degradation (Momoi & Lennon, 1982). Muscle from some patients may be ischemic and partially denervated, but by no means provides a consistent source of extra-junctional AChR (see below). The AChR content of human adult muscle extracts was variable, ranging from 0.23 - 2.19 pmol α-BGT binding sites/g muscle, which is within the range quoted by other workers (see Table 20). There are several possible reasons for the variability observed - proteolysis may occur between amputation and freezing of muscle at -80^{o}C, and although the duration of this interval was routinely less than one hour, the inadequate yields of AChR obtained from post mortem tissue supports the liklihood that post mortem deterioration occurs. It is also possible that muscle stored at -80^{o}C may lose its [^{125}I]α-BGT binding activity during the freeze-thaw cycle or during prolonged storage, however, there was no evidence to suggest that this was so. The most likely explanation for the variability of AChR yield is the inherent variation in the muscle samples used. Most muscle samples were obtained from patients suffering from vascular problems or diabetic gangrene, and it is likely that this tissue has varying degrees of ischemia with accompanying tissue autolysis. Also, most amputees were older than 60 years of age and motor innervation in such patients is reduced (Tomlinson & Irving, 1977). This, therefore, increases the liklihood of proliferation of AChR and production of a heterogenous population of AChRs in these muscle samples (Dolly, 1979). One muscle sample which could be classified as normal, innervated tissue (obtained from the victim of a road traffic accident) gave a yield of 0.39 pmol α-BGT binding sites/g muscle. Although this value is at the lower end of the range of values obtained, its interaction with anti-(AChR) antibodies in

Table 20. Yield of AChR from mammalian skeletal muscle types

Species	Yield AChR (pmol/g wet weight)	Reference
(Torpedo	> 1700	Eldefrawi et al., 1972)
Rat - innervated	8.5	Almon et al., 1974
	2.5	Berg et al., 1972
- denervated	23	Berg et al., 1972
	99	Dolly & Barnard, 1977
	60	Miledi & Potter, 1971
Chick - innervated	0.5 - 0.8	Sumikawa et al., 1982b
- denervated	14 - 31	
- embryonic	27 - 36	
Rabbit - innervated	0.35	Gotti et al., 1983
- denervated	4.87	
Cat - denervated	88	Dolly & Barnard, 1977
Calf - foetal	2.5 - 5.3	Gotti et al., 1982
	4.1	Einarson et al., 1982

Species	Yield AChR (pmol/g wet weight)	Reference
Human - adult		
-bone cancer	0.04 - 0.14	Mamoi & Lennon, 1982
	0.18 - 2.55	
-ischemic vascular disease	0.7	Stephenson et al., 1981
	0.23 - 2.19	this thesis
	2.7	
	0.9	Vincent & Newsom-Davis, 1979
-extraocular	0.8	
	2.0	
-cerebral disease	0.8	Vincent & Newsom-Davis, 1982b
-diabetic and peripheral neuropathy	4	
	2.25 - 5.12	
-malignant bone tumour	0.44 - 1.48	Lefvert, 1982
-myasthenic	0.12	
-road traffic accident	0.39	this thesis
Human - foetal		
-prostaglandin induced	0.6 - 1.9	this thesis

myasthenic serum was comparable with that of muscle samples giving higher initial yields of AChR (see "Results" section 6.2).

Foetal muscle is another source of human AChR, and generally represents normal, undiseased muscle. The yields of AChR obtained from such tissue (0.61 - 1.89 pmoles [^{125}I]α-BGT binding sites/g muscle, mean value $\pm$ SE (n) = 1.20 $\pm$ 0.10 (15)) were fairly constant over the age range studied (see "Results" section 6.1), and were 50% higher than those obtained for adult muscle. In view of the fact that AChRs are present over the entire surface of developing myotubes (Bevan & Steinbach, 1977), the yields of AChR in foetal muscle might have been expected to be much higher than those for adult AChR. However, denervation of adult muscle resulting in proliferation of receptors may explain these results.

Detergent extracts of rat muscle AChR provided a much more consistent yield of AChR (see "Results" section 2.3), probably representing a more homogenous source of AChR types.

The method used for purification of AChR was essentially the same as that described by Stephenson et al. (1981), which is a modification of methods described by Wonnacott et al. (1980) for the purification of Torpedo AChR. The method aims to reduce proteolysis effects by reducing the time taken for the preparation to a minimum, maintaining all operations, where possible, at 4^{o}C, and including a broad spectrum of protease inhibitors and anti-bacterial agents. The use of anti-proteases has been shown to improve the recovery of intact proteins upon purification (Silman et al., 1978; Dolly & Barnard, 1975; Lindstrom et al., 1979a) with the proviso that this is accompanied by accelerated purification times and use of low (4^{o}C) temperature. The presence of lysosomal enzymes has been

demonstrated in muscle cells of immature animals (Gutmann et al., 1976), adult rat diaphragm (Parsons & Pennington, 1976) and human muscle (Bury & Pennington, 1975), and it is therefore likely that after homogenization and extraction of muscle, proteolytic enzymes are released. For this reason a number of anti-proteases were added (see Table 21).

The use of all these measures permitted the isolation of purified adult AChR with specific activities within the range of values quoted in the literature (see Table 22). Values for adult human AChR were 0.01 - 0.8 μmol α-BGT binding sites/g protein, and for foetal AChR were 0.01 - 0.1 μmol α-BGT binding sites/g muscle; compared with a range of 0.19 - 11 μmol/g reported by other workers. The relatively low specific activities may be due to measurement of non-receptor protein, and although co-purification of receptor and non-receptor proteins is possible, it is more likely to result from an over-estimation of the protein concentration, which in most cases was at the lower end of the sensitivity of the assay. Also, since the receptor is an integral membrane protein with bound detergent (Meunier et al., 1972b; Karlin et al., 1979), it may behave differently from the standard protein used in the assay. The function of the receptor may be altered during the purification procedure, which may alter its ability to bind [^{125}I]α-BGT. It is known that Triton X-100 inhibits receptor function (Fischbach & Lass, 1978), so solubilization of the receptor may contribute to loss of activity. The receptor is also exposed to high concentrations of agonist (carbachol) during elution from the α-toxin affinity column, and this is thought to cause desensitization of the receptor, accompanied by a slow change in conformation and

Table 21. Mode of action of protease inhibitors

Inhibitor	Mode of action	Reference
EDTA	Inhibits calcium-activated proteases	Dayton et al., 1976
Benzethonium chloride	Bacteriocide	Povl Elo, 1963
PMSF	Irreversible block at active site of serine esterases	Gold, 1967
Sodium azide	Inhibits bacterial growth	
Bacitracin	Antibiotic polypeptide from Bacillus subtilis and licheniformis	Desbuquois et al., 1974
Benzamidine	Inhibits conversion of collagen to procollagen	Byers et al., 1975
Pepstatin	Blocks lysosomal proteases	Schwartz & Bird, 1977

Table 22. Specific activities of purified AChR from mammalian skeletal muscle

Species	Specific activity (pmol/μg)	Reference
Rat - innervated	0.19	Brockes & Hall, 1975a
	2.1, 6.4	Kemp et al., 1980
- denervated	0.53	Brockes & Hall, 1975a
	2.8 - 7.5	Kemp et al., 1980
Chick - innervated	-	
- denervated	6	Sumikawa et al., 1982
- embryonic	4 - 6	
Rabbit - denervated	4.1	Gotti et al., 1983
Cat - denervated	10 - 11	Shorr et al., 1978; 1981
Calf - foetal	1.5 - 2.5	Lindstrom et al., 1979b
	6	Gotti et al., 1982
	7.5	Einarson et al., 1982
Human - adult	5 - 7	Momoi & Lennon, 1982
	0.5 - 3.5	Stephenson et al., 1981
	0.01 - 0.8	this thesis
Human - foetal	0.01 - 0.1	this thesis

loss of ability to bind [^{125}I]α-BGT (Heidmann & Changeux, 1979). This may account for the low (less than 20%) recovery of [^{125}I]α-BGT binding activity after the α-toxin affinity chromatography stage, although the use of much lower concentrations of the more potent agonist benzoquinonium chloride instead of carbachol as the competitor does not lead to better recoveries (Gill Turnbull, personal communication). The absorption of AChR from muscle extracts to immobilized α-toxin was found here to be 90 - 95%, which is greater than that obtained by Mamoi & Lennon (1982), who routinely absorb 52% AChR and Gotti et al. (1982) who find 49 - 66% absorption of AChR. The subsequent recovery of AChR from α-toxin beads found here is also higher (20%, compared to 10%), which may be a reflection of the higher degree of substitution of α-toxin onto the Sepharose-4B beads used here.

The very low specific activities obtained for purified human foetal AChR can possibly be attributed to its unstable toxin binding activity (see "Results" section 2.2), both in detergent extracts and in purified preparations, which may result from the high levels of proteases present in foetal tissue, as discussed above. Reduced amounts of lipids present in foetal tissue compared with adult (Hall, 1981) may also contribute towards this observation. Lindstrom and co-workers (1980b) have shown that the continual presence of soybean lipid in mixed micelles, with cholate, is required to prevent irreversible inactivation of the cation channel in Torpedo. Greater instability of α-BGT binding sites in foetal human brain as compared with adult rat has also been observed (Sue Wonnacott, personal communication). Instability of foetal AChR compared with adult AChR may reflect structural differences between the two

receptor types, since stabilization of AChR does not accur until synaptogenesis is complete (Changeux & Danchin, 1976; Anderson, 1983) and may occur as a result of post-translational modifications (see "Introduction" p. 17).

Radioimmunoassay of anti-(AChR) antibodies

The radioimmunoassay for detection of anti-(AChR) antibodies uses [^{125}I]α-BGT - labelled human AChR as antigen, which is used to precipitate anti-(AChR) antibodies in myasthenic serum, and this complex is then precipitated using a second antibody (see "Introduction", Figure 6). Basic radioimmunoassay procedures have been reported by a number of groups of workers (Lindstrom et al., 1976a; Lindstrom, 1977; Monnier & Fulpius, 1977; Lefvert et al., 1978; Newsom-Davis et al., 1978; Dwyer et al., 1979; Tindall et al., 1981). However, the assay comprises several stages which engenders considerable variations in some aspects of the published procedures, particularly the exact experimental conditions. The procedure described here optimizes the conditions of the radioimmunoassay, providing an overall method which is flexible, reproducible and suitable for routine application, and can, if necessary, supply a speedy determination for the clinician.

Maximal formation of labelled antigen was achieved by incubating a 10-fold molar excess of [^{125}I]α-BGT with AChR, after which, incubation of labelled toxin-AChR complex with myasthenic sera was performed, and found to be complete after 16 h at 4^{o}C or 2 h at 23^{o}C. Incubation of [^{125}I]α-BGT-AChR-antibody complex with goat anti-(human IgG) was similarly complete after 16 h at 4^{o}C or 2 h at 23^{o}C. Titres fell slightly when incubation times of greater than 48

h were used. Serial dilutions of sera were made in order to ensure sufficient molar excess of antigen, and ratios of 3:1 were found to be sufficient to ensure maximal precipitation of anti-(AChR) antibodies. Maximal formation of receptor-antibody complexes requires also a minimum concentration of AChR which depends upon the the dissociation constants of the receptor-antibody complexes. A value of 0.5 nM [^{125}I]α-BGT binding sites was sufficient to ensure maximum formation of complex, although higher concentrations of receptor may well be advantageous in the case of some sera for which 0.5 nM may be a minimum acceptable figure.

Sufficient antigen concentration of extracts having only low amounts of AChR could be achieved by vacuum dialysis concentration, so that most detergent extracts could be utilized in the radioimmunoassay. AChR extracts concentrated in this way showed an increase in the non-specific binding of [^{125}I]α-BGT, but nevertheless appeared to lead to accurate antibody titres.

Although there has been one report (Bradley et al., 1978) that the use of rat muscle AChR as an antigen in the radioimmunoassay gives the same anti-(AChR) antibody titre as when purified human AChR is used, most other groups find that the use of rat AChR as antigen gives lower antibody titres than when human AChR is used, and the percentage of positive results is generally lower (60 - 70%, compared with 80 - 90% for human - Mittag et al., 1981; 1984; Savage-Marengo et al., 1979; Kornfeld et al., 1981). The use of muscle from other mammalian species as an alternative source of AChR is not to be recommended as, although such tissue may give a higher, more consistent yield of AChR, it is clear that these, too, give rise to generally lower anti-(AChR) antibody titres when used

in the RIA (Roses et al., 1981; Mickle et al., 1983; Gotti et al., 1983; Williams et al., 1984).

The procedure for radioimmunoassay of anti-(AChR) antibodies outlined in the "Methods" (section 8) allows the determination of reproducible titres. Repeat determinations on an individual serum sample using a single extract of AChR and freshly prepared [^{125}I] α-BGT gave values of 2.99 $\pm$ 0.04 nM [^{125}I]α-BGT binding sites ($\pm$SE, 15 assays). Repeat assays of the same serum sample using a single extract of AChR and 4 different preparations of [^{125}I]α-BGT gave values of 3.23 $\pm$ 0.08 nM [^{125}I]α-BGT binding sites ($\pm$SE, 15 assays). Repeat assays of a different serum sample using a single preparation of [^{125}I]α-BGT and 4 different AChR extracts gave values of 1.80 $\pm$ 0.07 nM [^{125}I]α-BGT binding sites ($\pm$SE, 10 assays). Thus despite the variability resulting from different [^{125}I]α-BGT and AChR preparations, values differed by less than 15%. Titres obtained for a given serum sample using a single AChR extract and sample of labelled toxin changed little over a period of 2 weeks. Storage of labelled toxin for periods of longer than 3 weeks led to much greater variation in antibody titres and toxin was therefore not used beyond this point. There was no evidence of systematic variations in antibody titres of given serum samples arising from changes in AChR extracts on storage at 4^{o}C for up to 3 months. The consistency of assays obtained by using single [^{125}I]α-BGT and AChR preparations suggests that there may be some advantage in storage of serial serum samples taken from an individual patient over a period of treatment in order to assess most accurately the effect of such treatment on anti-(AChR) antibody titres. However, changes of antibody titre resulting from plasma exchange and immunosuppression

are generally much greater than differences resulting from the use of different [^{125}I]α-BGT and AChR preparations.

Obviously it would be desirable to have a directly labelled antigen, rather than having to rely on an external ligand because the occupancy of the ACh binding site by [^{125}I]α-BGT excludes measurement of antibodies directed at this site. These factors are discussed more fully later on (see "Discussion" p. 233).

Specific features of antibody patterns in myasthenia gravis

The radioimmunoassay for anti-(AChR) antibodies discussed above provides a useful aid to the diagnosis of MG, since approximately 80 - 90% of clinically diagnosed myathenic patients have antibody titres which are elevated above normal controls, when assayed using human AChR as the antigen (Lindstrom et al., 1976a; Seybold et al., 1981). This value is reduced to 60% if rat AChR is used as the antigen in the assay (Mittag et al, 1981; 1984). Antibody titres obtained from serial assays on serum samples from a given myasthenic patient can, in many cases, reflect the clinical state of the patient, an observation which has been most convincingly demonstrated in serial samples obtained from patients undergoing plasma-exchange coupled with immunosuppressive therapy (Newsom-Davis et al., 1978; 1979; Carter et al., 1980). Plasma exchange has become an increasingly popular treatment in many immunopathological diseases (eg. demyelinative neuropathies, inflammatory myopathies, Lambert-Eaton Myasthenic syndrome, multiple sclerosis etc.). Firm evidence for the therapeutic benefits of plasma-exchange in neurological disorders is limited to MG, but even

here no large scale randomized controlled trials have been performed, and this form of treatment tends to be confined to patients undergoing myasthenic crisis or to those with rapidly progressing severe disease which is unmanageable by drug treatment or thymectomy alone (Lisak, 1984; Dau, 1984). The effects of plasma-exchange, which can produce a rapid and marked clinical improvement, are usually associated with a sharp fall in anti-(AChR) antibody titre and have been reported by a number of groups of workers (Pinching et al., 1976; Dau et al., 1977; Behan et al., 1979). However, such benefits are usually short term, and the long term response is no better than that produced by medication alone (Newsom-Davis et al., 1979; Hawkey et al., 1981; Keesey et al., 1981).

Across a range of patients the correlation between anti-(AChR) antibody titre and severity of clinical symptoms is poor (Lefvert et al., 1978; Barkas et al., 1979; Vincent & Newsom-Davis, 1979), suggesting that this radioimmunoassay might not accurately reflect levels of particular antibodies with immediate relevance to the disease state. Convincing evidence for an important, although not necessarily primary role for AChR in the aetiology of MG is provided by the observation that injection of experimental animals with xenogeneic AChR leads to EAMG (Patrick & Lindstrom, 1973). It is conceivable that the parts of the receptor molecule most likely to be conserved between species are those most relevant to the functioning of the receptor and that antibodies directed against such determinants could represent subclasses with most significance in the disease state. However, the use of alternative mammalian AChR species as antigens in the RIA, although consistently giving lower

antibody titres than when human AChR was used as antigen, gives no better correlation with the disease state (Savage-Marengo et el., 1979; 1980; Harrison et al., 1981; McAdams & Roses, 1980; Mittag et al., 1981; 1984).

Patient-specific antibody patterns

The presence of discrete sub-populations of anti-(AChR) antibodies in myasthenic sera are clearly reflected by the differing anti-(AChR) antibody titres obtained by using AChR preparations from different species. Many groups of workers have demonstrated heterogeneity of anti-(AChR) antibodies by using different assay methods (Mittag et al., 1978; Lindstrom et al., 1978a; Vincent & Newsom-Davis, 1979; Weinberg & Hall, 1979; Mittag et al., 1981; Lefvert et al., 1981; Garlepp et al., 1981; Tindall, 1981; Barkas et al., 1982; Zielinski et al., 1982; Vincent et al., 1983; Whiting et al., 1983); but reports of the extent to which patterns of antibody diversity are constant and characteristic of an individual myasthenic patient have been limited (Garlepp et al., 1981; Tzartos et al., 1982; Kalies et al., 1984).During the course of investigations using rat AChR antigens to try to correlate anti-(AChR) antibody titre with the severity of the disease, it was found that the ratio of anti-(rat AChR) antibodies to anti-(human AChR) antibodies varied between patients but was constant for a given patient over the period of study (Savage-Marengo et al., 1980). The results in this thesis (see "Results" section 4.2) have confirmed and extended this study. A proportion of samples from 6 of the patients studied are those given in Savage-Marengo et al., 1980; a further number are given in Harrison et al., 1981.

Anti-(AChR) antibody titres were determined, using junctional rat, extra-junctional rat and human AChRs as antigens, in sera from 14 MG patients, all of whom experienced a range of clinical states and widely varying anti-(human AChR) antibody titres while undergoing plasma-exchange and immunosuppression therapy during the period of investigation (Behan et al., 1979; Carter et al., 1980). In agreement with Weinberg & Hall (1979) it was found that the titres obtained with junctional rat AChR as antigen in the radioimmunoassay were always lower than those obtained by using extra-junctional AChR. The relatively constant proportions of both anti-(junctional rat AChR) and anti-(extra-junctional rat AChR) antibodies to anti-(human AChR) antibodies in individual patients over periods of time upto five years, despite fluctuating antibody titres, suggests that the overall pattern of antibodies is specific for that patient. This view is supported by the consistency of the percentage precipitation of AChR shown by each patient (see "Results" section 4.2 & 4.3) which provides further support for a patient-specific antibody pattern. The constancy of the patterns observed suggests that the detailed nature of the response is characteristic of a given patient and can be most simply explained by an initial limited exposure of the AChR antigen to the immune system, rather than a repeating response to a changing modified receptor which might result, for example, from the shedding of receptor from the muscle surface. In the latter case the antibody pattern would be expected to vary with each presentation of the antigen to the immune system.

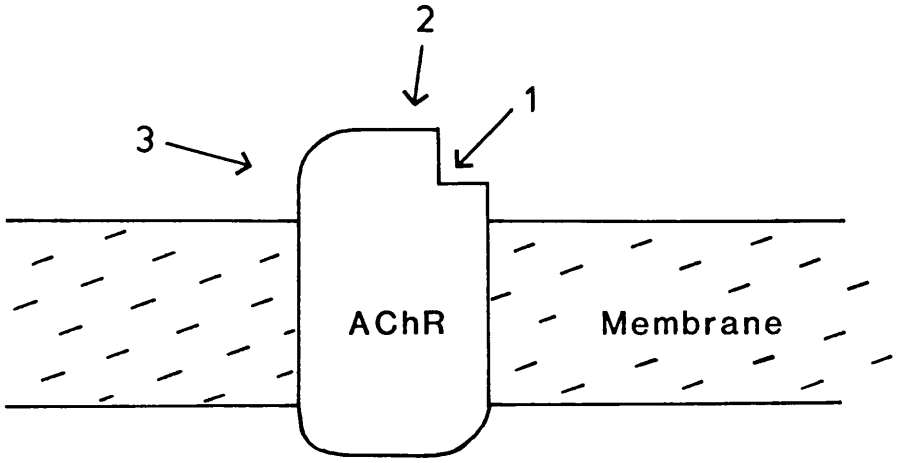
Percentage precipitation of AChR by excess myasthenic serum

The relative abilities of different myasthenic sera to precipitate [^{125}I]α-BGT labelled AChR complexes (see "Results" sections 4.2 & 4.3) were also found to be specific for a given patient. These findings can be explained in terms of heterogeneity of toxin binding components coupled with the presence of discrete sub-populations of antibodies in myasthenic sera. However, the results of combining myasthenic sera (see "Results" section 4.3.1) argue against the suggestion that increased precipitation of AChR by certain sera is caused by absence of particular antibody sub-populations.

An alternative explanation involves the presence of antibodies that cause release of bound [^{125}I]α-BGT, such antibodies may or may not represent what other workers report as anti-(α-BGT binding site) antibodies ("anti-site" antibodies). In the commonly used radioimmunoassay procedure, the ACh binding site is blocked by the presence of [^{125}I]α-BGT, so precluding the measurement of these antibodies (see Figure 54). The possible implications of such a class of antibodies is discussed in the "Introduction" (p. 25).

"Anti-site" antibodies were first described by Almon et al. (1974a); many groups since then have attempted to show conclusively that such antibodies are present in myasthenic sera, and whether or not they are relevant to the disease state. Electrophysiological studies have shown that serum or IgG from patients with MG (Anwyl et al., 1977; Bevan et al., 1977; Harvey et al., 1978) and animals with EAMG (Patrick et al., 1973) cause reduced sensitivity of muscle cells in culture to applied ACh. Inhibition of α-BGT binding to normal human muscle was shown in 75% of patients by histochemical

Figure 54. Schematic representation of possible sites of attachment of anti-(AChR) antibody causing 'blockade' of the AChR



1. The antibody attaches to the ACh binding site

2. The antibody attaches near the ACh binding site and sterically hinders the binding of ACh or α-BGT

3. The antibody does not directly interfere with the ACh binding site, but alters the AChR molecule, thus inhibiting the ACh or α-BGT binding site.

means (Bender et al., 1975; 1976). More recent studies have used immunoprecipitation methods, which are much more convenient than those mentioned above. Putative assays for "anti-site" antibodies involve quantitative evaluation of the competition between antibodies and α-BGT for binding to AChR from different sources. Such assays have revealed that sera from 7 - 90% of MG patients contain "anti-site" antibodies.

A few groups of workers have attempted to show some relationship between "anti-site" antibodies and clinical state. Vincent & Newsom-Davis (1979) in a study of 73 myasthenic patients showed that 65% of these had "anti-site" antibodies. They were unable to relate the presence of such antibodies to the clinical state of the patients, but did show that no patients in remission had significant "anti-site" antibodies although many had high total anti-(AChR) antibody levels. Zurn & Fulpius (1977), using rabbits immunized with electric fish AChR, found that levels of "anti-site" antibodies were increased seven-fold in rabbits with myasthenic paralysis. Fulpius et al. (1980) later showed the presence of "anti-site" antibodies in 4 MG patients, detailed analysis of one of these patients revealed that antibody binding to chicken embryo myogenic cultures competed with [^{125}I]α-BGT and cholinergic ligands, and that binding of this specific antibody accelerated degradation. These investigations were subsequently extended (Fulpius et al., 1981) and studies on a group of 21 MG patients revealed that IgG purified from 10 of these patients showed varying degrees of inhibition of [^{125}I]α-BGT binding to chick muscle - these patients were those with the most severe myasthenic symptoms. Drachman et al. (1981) found that 88% of an unspecified number of MG patients have

immunoglobulins which block α-BGT binding to cultured muscle cells. This blockade was accompanied by an acceleration of receptor degradation, and was found also to correlate with the severity of the disease in these patients. Desouki et al. (1981) found that IgG fractions purified from antisera from rabbits and sheep immunized with Torpedo AChR inhibited AChR function by interfering with the agonist-induced conformational changes in the receptor-channel complex, and, in addition, that immune rabbit IgGs also inhibited [^{3}H]ACh binding to the receptor. These antibodies would therefore inhibit receptor function by blocking its conformational changes without occupying the agonist and antagonist binding sites.

Mittag et al. (1984) suggest that the high percentage of MG patients who do not have an anti-(AChR) antibody titre may reflect the presence of exclusively "anti-site" antibodies in these patients. Although present at low concentrations (0.1 - 0.7 nM, compared with 0.5 - 130 nM for antibodies not directed at the α-toxin binding site), such antibodies may contribute significantly to the pathological condition. One obvious way of looking for "anti-site" antibodies is to radiolabel the receptor directly, thus leaving the ACh binding site free, after which it may be possible to detect antibodies which were previously unable to bind to the receptor. Torpedo AChR has been successfully iodinated (Aharonov et al., 1977; Lindstrom et al., 1981) and has been used for probing anti-(AChR) antibodies (Souroujon et al., 1983; Moshly-Rosen et al., 1979). Preliminary attempts to iodinate purified human adult AChR by using the standard chloramine-T method used for iodination of -BGT (see "Methods" section 1.1) were not very promising. Incorporation of [^{125}I] into the protein was only 2%, the biological activity

(assessed by its ability to bind to myasthenic serum) was less than or equal to 1%, and this, coupled with high background counts meant that the AChR labelled by this method was not useful for probing MG sera. Further investigations along this line would necessitate the development of a more gentle iodination procedure which would yield labelled AChR of much higher biological activity. It would also be desirable to increase the yield of AChR obtained from elution after the affinity chromatography stage of AChR purification as the yield of purified receptor obtained for iodination under the existing conditions is also a limiting factor. The success of using iodinated Torpedo AChR stems from the high yield of protein obtained from its purification.

The production of rat monoclonal antibodies (raised against Torpedo AChR) (Lennon & Lambert, 1980) which bound to muscle AChR at a site removed from the binding site for cholinergic ligands, and which caused impairment of neuromuscular transmission in mice, rats and guinea-pigs suggested that "anti-site" antibodies may not be the major pathogenic agent in MG. Many groups since then have produced monoclonal antibodies, some of which, like the polyclonal antisera of Zurn & Fulpius (1977), produce pharmacological blockade of AChR (Gomez & Richman, 1983; Souroujon et al., 1983); and others, like those of Lennon & Lambert (1980) which bind at a site removed from the ligand binding site and still induce EAMG (Richman et al., 1980). There is still, therefore, much controversy regarding the involvement of "anti-site" antibodies, and their importance in the pathogenesis of MG remains unclear (Vincent, 1980; Fulpius, 1983; Drachman, 1983).

The present results (see "Results" sections 4.2 & 4.3) showed that myasthenic sera vary in their ability to precipitate a limited amount of [^{125}I]α-BGT-labelled AChR, from 15 - 100%. Sera from MG patients which precipitated low amounts of toxin-receptor complex did not show appreciable inhibition of α-BGT binding to the receptor - one observation that indicates that "low-precipitating" sera may effect loss of labelled toxin by a mechanism other than inhibition of toxin binding, for example by binding to AChR at a determinant removed from the ACh binding site and which may be similar to the observations of Lennon & Lambert (1980) and Richman et al. (1980) described above. Binding at such a site may promote a conformational change which causes accelerated release of [^{125}I]α-BGT. The results obtained here (see "Results" section 4.3) show that some MG sera are capable of accelerating the release of bound [^{125}I]α-BGT from its receptor complex, and that such sera are those which give low values for the percentage precipitation of AChR by excess myasthenic serum (see Figure 30, Table 13). The slow rate of release of [^{125}I]α-BGT in the presence of "high-precipitating" sera, normal serum or buffer can be explained by assuming that [^{125}I]α-BGT is bound tightly to the AChR, but even so, will slowly dissociate. The dissociation curve obtained in the presence of an excess of unlabelled α-BGT is similar to that obtained from kinetic experiments for determining the dissociation constant (k_{-1}) (see "Results" section 5.3.2), where rebinding of the radioactive ligand at equilibrium is prevented by the presence of excess cold ligand.

Reports of accelerated dissociation of [^{125}I]α-BGT from toxin-receptor complexes have also been reported by other workers using polyclonal antisera and monoclonal antibodies. Barkas &

Simpson (1982) and Kalies et al. (1984) find that antisera or IgG from MG patients can displace bound toxin from the receptor, Boulain & Menez (1982) have similarly demonstrated accelerated dissociation of neurotoxin from AChR brought about by the binding of polyclonal and monoclonal antibodies to cobra venom and Naja nigricolis toxins, respectively. Two monoclonal antibodies raised against chick muscle AChR were found to inhibit the reaction of α-BGT with homologous AChR, although these antibodies were not directed precisely to the neurotoxin binding site of the receptor (Mehraban et al., 1984), polyclonal antisera exhibiting such an ability have also been reported (Dolly et al., 1983) and in these cases the observed blockade has been attributed to a steric or conformational constraint exerted by IgG molecules. One of the monoclonal antibodies raised against chick muscle AChR was able to cause rapid dissociation of toxin-receptor complexes ($T_{\frac{1}{2}}$ = 0.5 h at 23°C, and this has again been attributed to an antibody-induced conformational change in the AChR oligomer (Mehraban et al., 1984).

However, using the methods described here, it is not possible to distinguish between (i) competition for an identical site (ii) steric hindrance from an adjacent site or (iii) a conformational change from binding to a distant site, in the inhibition or release of [^{125}I]α-BGT binding to AChR by anti-(AChR) antibodies. Furthermore, α-BGT is much bigger than the native ligand (α-BGT has MW of 8000, compared with 182 for ACh) and may not bind at the ACh site, so this can confuse the issue and it remains to be seen whether an α-BGT displacing antibody may be relevant to the physiology of MG.

Clearly, the development of suitable methods for the quantitation and characterization of "anti-site" and anti-(α-BGT) displacing antibodies is still required before their importance in the disease state can be fully assessed.

Inhibition factor

The presence of an "inhibition factor", which apparently inhibited the AChR-antibody interaction, was found in the sera of approximately 20% of MG patients. Preliminary investigations suggested that this factor was a serum component, as IgG fractions from such sera did not display the same characteristics. In order to study this phenomenon further, it may be necessary to find out at which stage of the radioimmunoassay this "inhibition factor" has its effect - at the interaction of labelled AChR with anti-(AChR) antibody or at the interaction of AChR-antibody complex with goat anti-(human IgG). Inhibition of antibody binding to AChR by α-fetoprotein *in vitro* has been reported (Abramsky & Brenner, 1979; Brenner et al., 1980; Brenner & Abramsky, 1981). The levels of α-fetoprotein are increased above normal levels in pregnant women, especially those in the latter term of pregnancy, and it has been suggested that α-fetoprotein protects the neonatal neuromuscular junction from maternal antibodies. The "inhibition factor" observed here was not, however, confined to sera from women only. Among the non-pregnant population, raised serum α-fetoprotein levels are also found in most cases of primary hepatoma and non-seminatomous germ cell tumours (Ruoslahti & Hirai, 1978). However, other workers have been unable to reproduce the inhibition observed by Brenner and co-workers (Tindall, 1981; Lefvert & Osterman, 1983; Heininger et

al., 1984), suggesting that α-fetoprotein may not be important as a protective agent in neonatal myasthenia gravis, or, indeed, as the "inhibition factor" observed here.

Comparison of the interaction of human adult and foetal skeletal muscle AChRs with [^{125}I]α-BGT and anti-(AChR) antibodies

In view of the reported similarities between extra-junctional and embryonic AChR (Burden, 1977a; Schuetze et al., 1978) and the finding by Weinberg & Hall (1979) that serum from one myasthenic patient showed the same antibody titre whether extra-junctional rat AChR or AChR from 18-day old rat embryos was used as an antigen in the assay, it is possible that the composition of anti-(AChR) antibodies in myasthenic sera could reflect an early exposure of embryonic AChR to the immune system. This is obviously pertinent to questions concerning the aetiology of MG.

Immunohistochemical studies on human adult and foetal muscle

Preliminary studies for the comparison of adult and foetal AChRs employed histochemical techniques, which have been used extensively to study the distribution of AChRs over the surface of muscle cells (Daniels & Vogel, 1975; Moody-Corbett & Cohen, 1981; Steinbach, 1981; Ishikawa & Shimada, 1981; 1982). These studies complement those of electrophysiological methods where membrane potentials are recorded during localized iontophoretic application of ACh to the cell surface. The similarities observed by other workers between embryonic and extra-junctional AChR suggested that in foetal muscle AChRs are distributed over the cell surface, contrasting with the distribution of AChRs in adult muscle, where

they are localized at the synapse (see Introduction p. 12).

Staining of human adult and foetal muscle sections with α-BGT using the 'PAP' technique were disappointing, the high background staining seen with the rabbit anti-(α-BGT) antiserum (see "Results" section 5.1) meant that no conclusions could be drawn from these studies. Fluorescein staining, although offering no amplification, provides a direct method for visualization of receptors. Teased human adult muscle fibres showed staining throughout the fibre, with occasional areas of more intense staining. The pattern of staining did not correspond with that observed by Ringel et al. (1975) who found that AChRs were clustered in the post-synaptic folds of the muscle fibre. Foetal muscle fibres showed weak staining along rows of regularly arranged cells, which may represent developing muscle fibres (Minguetti & Mair, 1982). The distribution of AChE did not correspond with the pattern of fluorescein staining observed - there was no significant staining for AChE on human adult muscle fibres, which may result from a decrease in levels of AChE in ischemic muscle from older patients (Bourgeois et al., 1978; Betz et al., 1980). Staining for AChE in foetal muscle fibres, on the other hand, was observed all over the surface of the fibres.

These preliminary attempts to investigate the distribution of AChR in human adult and foetal muscle were inconclusive and were not pursued; however, a detailed study could be enlightening. Alternative methods for comparing adult and foetal AChRs were then studied, namely their interactions with α-BGT and anti-(AChR) antibodies from myasthenic sera.

[^{125}I]κ-BGT binding characteristics of AChRs in detergent extracts of human adult and foetal skeletal muscle

The kinetic constants for association, dissociation and equilibrium binding of [^{125}I]κ-BGT to AChR were determined. For a receptor binding experiment:-

$$R + L + I \rightleftharpoons RL + RI$$

where:- R = concentration of free binding sites

I = concentration of unlabelled drug

RL = concentration receptor bound to radioactive ligand

= parameter measured

RI = concentration receptor bound to unlabelled drug

L_T = total ligand concentration

= L + RL

L = concentration free radioactive ligand

3 basic types of experiment are usualy performed within this framework:-

1. Kinetic experiments in which RL is determined as a function of time with L_T held constant
2. Saturation experiments in which L_T is increased and RL determined at equilibrium
3. Inhibition experiments in which RL is determined as the concentration of unlabelled drug is increased and L_T is held constant.

There are many reports in the literature for such experiments on the kinetic and equilibrium binding of radiolabelled curare-mimetic neurotoxins with n-AChR from central and peripheral tissue (Lukas et al., 1981). Direct comparisons of data obtained from within and between species are often difficult because of the

Table 23. Kinetic constants for the reaction from innervated and denervated

Muscle type	α-BGT species	Temp. (°C)	k_{+1} (x 10^5 $M^{-1}s^{-1}$)
Rat diaphragm			
- innervated	mono-	35	1.0, 0.3
- denervated	iodinated	35	3.1, 1.2
Cat leg (9s)			
- denervated	[^{3}H]	25	1.8
Chick embryo			
- 11 day old	[^{3}H]	25	0.54
Human adult			
- extraocular		23	1.3
- diabetic with peripheral neuropathy	di-iodinated	23	2.4
- ischemic calf		23	1.9
- ischemic calf (purified)	unfractionated	23	3.46
- ischemic leg	unfractionated	23	5.74
Human foetal	unfractionated	23	8.61

of α-BGT with detergent extracts of AChR

mammalian skeletal muscle

k_{-1} (s^{-1}) ($\times 10^{-4}$)	($\times 10^{-6}$)	K_D ($\times 10^{-9}$ M)	Reference
0.63	< 1.9	0.63, < 0.06	Brockes & Hall, 1975a
0.63	< 1.9	0.20, < 0.02	
not detectable after 50 h			Dolly et al., 1981
0.19	-	0.0035	Lo et al., 1981
0.32	0.96	0.25, 0.006	Vincent & Newsom-Davis, 1979
0.48	1.2	0.20, 0.005	
0.32	0.96	0.17, 0.005	
0.024	-	0.69	Stephenson et al., 1981
0.66	10.2	0.91	this thesis
0.72	7.5	0.51	this thesis

different states of purification of receptor types, the degree of iodination of toxins, times and temperatures of incubations, etc., used for such determinations. Therefore, extensive comparison of binding data obtained here with those from the literature is not meaningful. However, Table 23 shows comparisons of binding data in detergent extracts from some mammalian muscle species. Some reports in the literature which compare the use of different iodinated α-BGT species have indicated that di-iodinated α-BGT differs from mono-iodinated and native α-BGT, especially in its rate of association with purified AChR (Wang & Schmidt, 1980; James et al., 1980). Lukas et al. (1981) also demonstrated anomalous biphasic dissociation kinetics using di-iodinated α-BGT and suggested that detailed binding studies should not be made using mixtures of the different iodinated α-BGT species. The routine preparations of [^{125}I]α-BGT used here comprise greater than or equal to 70% mono-iodinated α-BGT (see "Results" section 1.2), and as the aim of these experiments was to compare the binding characteristics of foetal and adult AChR, rather than to undertake a detailed kinetic study, unfractionated [^{125}I]α-BGT was considered to be suitable for these studies.

The binding data observed in the studies performed here showed no significant differences between the interaction of [^{125}I] α-BGT with AChR from human adult or foetal AChR. Scatchard analysis and Hill plots (see "Results" section 5.3) of the binding data indicate a single class of [^{125}I]α-BGT binding sites for both adult and foetal AChRs, with values for K_D in the nanomolar range. The observed mean equilibrium binding constants from Scatchard analysis (1.35 nM for adult and 0.67 nM for foetal) are higher than the true

K_D values obtained from the ratio of rate constants (0.91 nM for adult, 0.51 nM for foetal). This is to be expected because the apparent K_D obtained from equilibrium binding is equal to the true K_D (obtained from the ratio of rate constants) only if the concentration of binding sites used is much lower than the K_D, which, in a high affinity system, is difficult to achieve experimentally because of the lower limits of detection of radiolabelled ligands (Bylund, 1980; Cuatrecasas & Hollenberg, 1976). The values obtained for K_D are in the order of those reported by other workers for different mammalian muscle types (see Table 23).

Association kinetics between [^{125}I]α-BGT and AChR were rapid, and showed a single "on-rate". The results were analyzed by 2 methods:-

1. Pseudo first order kinetics, which take into account the reverse reaction
2. Second order kinetics, which uses initial rates of reaction (Dolly, 1979).

The values obtained by the 2 methods give good agreement (see "Results" section 5.3.1); the values obtained from the Pseudo-first order determinations are generally slightly lower than those obtained from second order determinations, which would be expected if the reverse reaction is significant.

In contrast to the results obtained by Stephenson (1980), the dissociation of [^{125}I]α-BGT from labelled adult and foetal AChR complexes gave biphasic kinetics with both fast and slow components. The faster initial phase accounted for 80%, and the slower phase for approximately 20%. This profile is similar to that obtained for

dissociation of [^{125}I]α-BGT from Torpedo membranes (Hanley, 1978). One would expect the equilibrium binding to be dominated by the low affinity site, although both fast and slow components dissociation rates should contribute directly to the measured K_D so that:-

$$K_D = \frac{k.K_H + K_L}{k_{+1}}$$

where:- K_D = equilibrium binding constant

K_H = dissociation constant for high affinity site

K_L = dissociation constant for low affinity site

K_{+1} = association rate

k = ratio of number of high to low affinity sites

(Oswald & Freeman, 1979)

Biphasic dissociation kinetics have also been reported by Vincent & Newsom-Davis (1979) for various human AChR preparations using di-iodinated α-BGT; and by Brockes & Hall (1975a) for rat junctional and extra-junctional diaphragm muscle using mono-iodinated α-BGT. Brockes & Hall (1975a) also document a biphasic association, and explain their results in terms of the presence of 2 independent classes of binding sites, although this does not exclude other interpretations, such as negative co-operativity between identical sites, or distinct sites that are in conformational equilibrium. In principle, these possibilities could be distinguished by rapid reaction techniques, but the minute amounts of AChR present in mammalian muscle preclude such studies. Lukas et al. (1981) find that biphasic dissociation of toxin-receptor complexes is more apparent when using iodinated α-BGT (especially di-iodinated α-BGT) rather than [^{3}H]α-BGT, and suggest

that alterations in receptor binding activity shown by di-iodinated α-BGT may result from alterations in the conformation of α-BGT induced by progressive iodination of an exposed tyrosine residue (Lukas(iewicz) et al., 1978). The presence of biphasic dissociation kinetics in rat brain (Brockes & Hall, 1975), human foetal brain (Whyte et al., 1984) and goldfish brain (Oswald & Freeman, 1979) has been attributed to the presence of two classes of binding sites while Maelicke (1977) explains the presence of biphasic dissociation by the presence of paired toxin binding sites which are interconvertible between a high and low affinity state. However, the studies performed here suggest the presence of one class of binding site only, although the presence of more than one can not be dismissed.

Interaction of human adult and foetal AChRs with anti-(AChR) antibodies in myasthenic sera and IgG

The first demonstration of a preferential interaction of myasthenic IgG with extra-junctional rather than junctional AChR was by Almon & Appel (1975), who showed that such IgG inhibited the binding of α-BGT to AChR from denervated, but not normal, muscle; and, in the radioimmunoassay, that denervated rat muscle was precipitated whereas normal muscle was not. This finding was confirmed and extended by Weinberg & Hall (1979) who showed that in radioimmunoassay of ten patients, anti-(AChR) antibodies measured using junctional or extra-junctional rat AChR as antigen differed with the antigen, and that the ratio of titres varied from 1.0 to 2.4. These observations indicate the presence on extra-junctional rat AChR of antigenic determinants recognized uniquely by myasthenic

sera. Anti-(rat AChR) antibodies, whether anti-(extra-junctional rat AChR) or anti-(junctional rat AChR), constitute only a small proportion of the total anti-(human AChR) antibody population (Savage-Marengo et al., 1979; 1980; Harrison et el., 1981), so the results above cannot necessarily be extended to the corresponding human AChR types. Furthermore, it is obviously much more relevant to a human disease to examine the composition of anti-(human AChR) antibodies, and for this reason the studies here were extended to investigate the interaction of foetal human AChR with anti-(AChR) antibodies.

Some evidence for a preferential interaction of different human muscle AChR types with anti-(AChR) antibodies in myasthenic sera is provided by two groups of workers. Vincent & Newsom-Davis (1979) compared "normal" adult human AChR (from patients with arteriosclerotic ischemia) with "denervated" adult human AChR (from patients with diabetic ischemia and peripheral neuropathy) as antigens in the radioimmunoassay for anti-(AChR) antibodies in myasthenic sera, and found essentially no differences between the two sets of titres. Subsequent work by the same authors (Vincent & Newsom-Davis, 1982b) made further comparative studies in which their "normal" AChR was derived post-mortem from a patient with cerebral disease, and their "denervated" AChR was obtained as previously. The observation here was that, in contrast to previous results, the antibody titres obtained using "normal" AChR were found to be variable, but slightly lower than those obtained using "denervated" AChR, and the authors suggested that the previously observed equality of titres may result from partial denervation of the ischemic "normal" muscle used in the earlier work. Alternatively,

post mortem changes to the "normal" muscle may lead to the variability observed.

"Denervated" AChR, again obtained from patients with diabetic ischemia and peripheral neuropathy, has also been compared with "normal" AChR, this time from muscle amputated from patients with malignant bone tumours, by Lefvert (1981; 1982), who found that in the radioimmunoassay, 40% of the myasthenic sera did not react with "normal" AChR, but of those that did, anti-(AChR) antibody titres were found to be approximately 50% lower than the corresponding titres obtained using "denervated" AChR as antigen.

The aim, therefore, of the comparison of human adult and foetal AChR in the radioimmunoassay was to show whether there was a preferential interaction of anti-(AChR) antibodies in myasthenic sera with foetal AChR. The results obtained (see Table 19) showed no significant difference between antibody titres obtained using adult or foetal AChR as antigen. Furthermore, the apparent K_Ds for binding of adult and foetal AChR to anti-(AChR) antibodies were identical, indicating that the affinity of antibodies directed against the two receptor types were the same. Like Vincent & Newsom-Davis (1979), the comparisons were made here largely by using "normal" AChR obtained from patients with severe vascular disease. Muscle from a healthy individual who had suffered a road traffic accident provided the opportunity to compare directly the binding of antibodies to this essentially normal AChR with that derived from patients with severe vascular disease; and it was found that again, the two muscle types showed virtually identical saturation curves (see Figures 51 - 53). So, while some muscle amputated from patients with vascular disease may be at least partially denervated, that used in the

experiments here can be regarded as providing antigenically normal adult human AChR with respect to its interaction with myasthenic serum.

The suggestion that foetal human AChR carries unique antigenic sites which are not present on normal adult AChR, and which are recognized by myasthenic sera, is not supported by the data reported here. It is possible, however, that such sites could have been lost during the purification procedure, especially in view of the fast initial degradation of the foetal receptor; or that the numbers of such corresponding antibodies in the sera tested are very low, and so beneath the limits of detection. Vincent & Newsom-Davis (1982b) reported, without data, that myasthenic sera reacted similarly with AChR from adult muscle or cultured foetal muscle, but their adult muscle was denervated and had previously given rise to higher antibody titres than their "normal" muscle. However the differences observed between the two types of adult muscle are not great, and also their foetal AChR is not directly comparable with that used here, which was obtained from foetuses aged between 14 and 22 weeks. It is possible that AChR obtained from younger or older foetuses might be antigenically different. It has been noted previously that sera from different myasthenic patients vary in their specificities, and in their ability to distinguish between junctional and extrajunctional rat AChR (see "Results" section 4.2; Savage-Marengo et al., 1980; Harrison et al., 1981). However, differential interactions between rat receptors are not necessarily reflected in the case of human receptors, since patients 14, 11 and 10 shown here have anti-(junctional rat AChR) to anti-(extra-junctional rat AChR) antibody titre ratios of 0.74, 1.00 and 0.16 respectively.

Structural studies of affinity purified adult and foetal human AChRs have revealed that the two receptor types are similar in that they both possess 4 subunits (apparent molecular weights 44000, 51000, 58000, 66000), they have similar isoelectric points and sedimentation coefficients. However, although in both cases all 4 subunits can be labelled with [^{125}I]-labelled Concanavalin A, the 44000 subunit of the foetal AChR binds less than the corresponding subunit of adult AChR, indicating a difference in the degree of glycosylation of the receptor (Turnbull et al., 1984). The observed differential interaction of MG sera with extra-junctional and junctional rat AChRs has been attributed to differences in the carbohydrate composition of the receptor types. Dwyer et al. (1981a) found, like Almon & Appel (1975) that antibodies from MG patients inhibit the binding of α-BGT to extra-junctional AChR more effectively than to junctional AChR. After glycosidase treatment of extra-junctional AChR, the inhibition was reduced, but not abolished, so that extra-junctional AChR more closely resembled junctional AChR (Dwyer et al., 1981a). They proposed that the observed differences between the two receptor types resulted from additional antigenic determinants on extra-junctional receptors which are located close to the toxin binding site, and which comprise carbohydrate. Antibodies from 1 MG patient have been reported (Hall et al., 1983) to recognize an α-BGT binding site on only extra-junctional or embryonic, but not on junctional rat AChR, these antibodies block toxin binding to solubilized Torpedo AChR by 50%. This blockade can be completely inhibited by N-acetyl glucosamine, but not by N-acetylgalactosamine, sialic acid, mannose, galactose or glucose. The authors suggest that the receptor

undergoes a change in glycosylation near one of the toxin binding sites during post-natal maturation of end-plates.

The relative absence of carbohydrate on the α-subunit of foetal human AChR compared with adult human AChR obtained by Turnbull et al. (1984) can be explained if one proposes that the carbohydrate serves to shield, rather than contribute to, antigenic sites in the immature form of the receptor.

It is therefore apparent that in order to draw any conclusions concerning the involvement of the foetal AChR as the auto-immunogen in MG, more extensive studies should be undertaken using a greater range of myasthenic sera and a wider age range of foetal muscle than has been the case in these studies. It is also possible that if an immunohistochemical technique could be established, the interactions of a range of myasthenic anti-(AChR) antibodies with a range of foetal receptors could be visualized and may well aid the immunological studies performed here.

CONCLUSIONS AND PROSPECTS

A careful study has been made of the steps involved in the radioimmunoassay for anti-(AChR) antibodies in myasthenic sera, and a reliable combination of experimental procedures has been developed, leading to a reproducible routine assay (Carter et al., 1981).

Using this assay, the levels of anti-(human AChR) antibodies, anti-(J-rat AChR) antibodies and anti-(EJ-rat AChR) antibodies have been monitored in 14 MG patients for periods of up to 5 years. These studies have confirmed and extended the findings of Savage-Marengo et al. (1980), and reveal that although the antibody titres of individual patients fluctuate widely during the period of investigation, the ratios of anti-(EJ-rat AChR) antibodies : anti-(human AChR) antibodies and the ratios of anti-(J-rat AChR) antibodies : anti-(EJ-rat AChR) antibodies are constant for a given patient during this time, and vary between patients from 0 - 0.6 and 0 - 1.0 respectively. Further evidence for this patient-specific antibody pattern was obtained from experiments where an excess of myasthenic serum was used to precipitate limited amounts of AChR. The precipitation of AChR varied between patients from 15 - 100% of that precipitated by serum from one particular patient, but was relatively constant for a given patient over extended periods of time. Such observations are most consistent with a single initiating event in the disease, rather than with a continued re-presentation of AChR antigen to the immune system (Harrison et al., 1981).

The mechanism of incomplete precipitation of AChR by myasthenic sera was investigated in two ways:- firstly, by combining

different sera together in an attempt to effect total precipitation of [^{125}I]α-BGT labelled AChR, which in no case led to any greater precipitation than that caused by the first serum alone. Secondly, attempts were made to show the presence of antibodies which might displace bound toxin from toxin-receptor complexes, by investigating the time-dependent displacement of toxin in the presence of myasthenic sera. The results obtained showed that some myasthenic sera are capable of accelerating the release of bound toxin from its receptor complex, and that this ability showed an approximately inverse relationship with the ability of the sera to precipitate [^{125}I]α-BGT labelled AChR. However, such observations do not distinguish between (i) competition for an identical site (ii) steric hindrance from an adjacent site or (iii) a conformational change from binding to a distant site. The present investigations would be complemented by the use of directly labelled purified human AChR, which, unlike [^{125}I]α-BGT labelled AChR, may have the capacity to detect anti-(toxin site) antibodies. The procedure for the purification of human AChR which is necessary for this approach is now a routine technique in the laboratory; however, the development of a gentle iodination procedure allowing full retention of biological activity is a necessary pre-requisite to such studies in order to achieve both sensitivity in the assay and a sufficient quantity of labelled protein.

The development of monoclonal antibody technology in recent years has significantly contributed to advances in the characterization of the AChR and its binding sites for anti-(AChR) antibodies. Several groups now have monoclonal antibodies to AChR

from electric fish and mammalian skeletal muscles, and have characterized the binding specificities of antibodies in MG sera by competition experiments (Tzartos, 1984). Such experiments could be performed here, in order to identify the nature of the binding of anti-(AChR) antibodies causing accelerated release of [^{125}I]α-BGT from [^{125}I]α-BGT labelled AChR complexes, and to identify sub-populations of antibodies in polyclonal sera which have particular relevance to the disease state.

In the course of studies using different muscle antigens in the radioimmunoassay, it was found that anti-(AChR) antibody titres obtained using EJ-rat AChR as antigen were consistently higher than when J-rat AChR was used, an observation made also by Weinberg & Hall (1979), who proposed that in view of the similarities between extra-junctional and embryonic AChR, embryonic AChR may constitute the autoimmunogen in MG. This has been investigated here by comparing the interaction of human adult and foetal AChRs with α-BGT and anti-(AChR) antibodies. The binding of α-BGT with tissue sections and teased muscle fibres from adult and foetal muscle were compared immunohistochemically using the 'PAP' technique and by fluorescein staining. The results of these immunohistochemical studies were disappointing and were largely inconclusive. However, a more detailed study could be enlightening, particularly if a control pattern of staining could be established beforehand, for example in rat muscle types. The use of monoclonal antibodies with varying specificities may again be useful probes for following the development of AChR in muscle fibres; furthermore such studies may clarify the similarities and differences between foetal,

extra-junctional and junctional receptors.

AChRs were isolated and partially purified from human adult and foetal muscle, and their binding to anti-(AChR) antibodies in myasthenic sera and IgG were compared. No significant difference was observed between the binding characteristics of the two receptor types, indicating the absence, at least in 14 - 22 week old foetuses, of ligand binding or antigenic sites unique to foetal AChR. This work should be extended to look at a wider range of foetal muscle types (including cultured foetal muscle), and also a wider range of myasthenic sera, if conclusions concerning the involvement of foetal AChR as the autoimmunogen in MG are to be drawn.

Differences in the interaction of rat muscle AChRs with myasthenic sera have been attributed to differing extents of glycosylation (Dwyer et al., 198la; Hall et al., 1983) of junctional and extra-junctional AChRs. Although such differences have not been observed here, it has been show that the 44000 molecular weight subunit of human foetal AChR is less heavily labelled with [^{125}I]Concanavalin A than the corresponding subunit of human adult AChR (Turnbull et al., 1984). Further studies on the carbohydrate composition of adult and foetal AChRs may give insight into structural differences between the two receptor types, and also into the pathways involved in post-translational modifications to the foetal AChR, which may be pertinent to the nature of its involvement in MG.

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PATIENT-SPECIFIC ANTI-ACETYLCHOLINE RECEPTOR ANTIBODY PATTERNS IN MYASTHENIA GRAVIS

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Assay of anti-acetylcholine receptor (anti-AChR) antibodies in the sera of myasthenic patients by the commonly used radioimmunoassay procedures [1-4] shows elevated antibody titers compared with the results obtained from non-myasthenic controls.[5] The assay accordingly is a useful aid to diagnosis of the disease. Nevertheless, the correlation of anti-human AChR antibody titer with the severity of clinical symptoms is poor [4, 6, 7] and we recently investigated the possibility that the use of rat AChR rather than human AChR as antigen in the radioimmunoassay might lead to a better correlation of disease state with antibody titer.[8] A crude rationale for such an approach can be found in comparison of the schematic diagrams of human and rat AChR shown in FIGURE 1. Both receptor molecules are depicted as having, in addition to the α-bungarotoxin-binding site, a limited number of antigenic determinants only some of which (two in FIGURE 1) are common to rat and human AChR. Myasthenic sera probably contain a range of antibodies, each specific for one of the antigenic determinants on human AChR, and assay of such sera by using an excess of human AChR as antigen might be expected to detect all these antibodies. Assays carried out by using rat AChR, on the other hand, should detect only those antibodies directed towards the common antigenic sites. In so far as it is reasonable to assume that the conserved sites are relatively important in the functioning of the receptor molecule, then antibodies directed towards those sites could represent subclasses with particular relevance to the disease state in myasthenia gravis.

Nine myasthenic patients representing a gradation of degrees of disease severity were chosen to compare the results of using rat and human AChR in the radioimmunoassay for anti-AChR antibodies. The titers obtained are shown in FIGURE 2 in which the patients A to I are arranged in order of increasing severity of disease. Two points clearly emerge from the data. Firstly the anti-(rat AChR) antibody titer is consistently lower than the anti-(human AChR) titer and secondly neither the anti-(human) nor the anti-(rat) antibody titers show any clear correlation with disease state.

The ratio of anti-(rat AChR) to anti-(human AChR) antibody titer showed considerable variation between patients. Nevertheless, assays on serial serum samples from individual myasthenic patients suggested that for a given patient the ratio might be relatively constant. We accordingly instituted a more com-

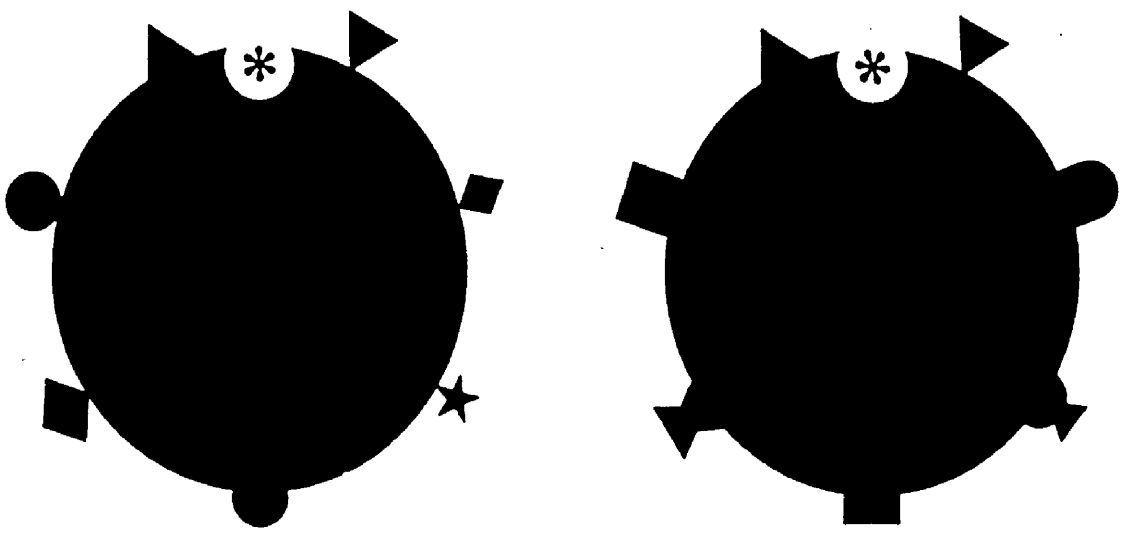


FIGURE 1. Schematic diagrams of human AChR and rat AChR. The asterisk represents α-bungarotoxin at its specific binding site. The remaining geometrical shapes represent exposed antigenic sites only two of which are shown as being common to human and rat AChR.

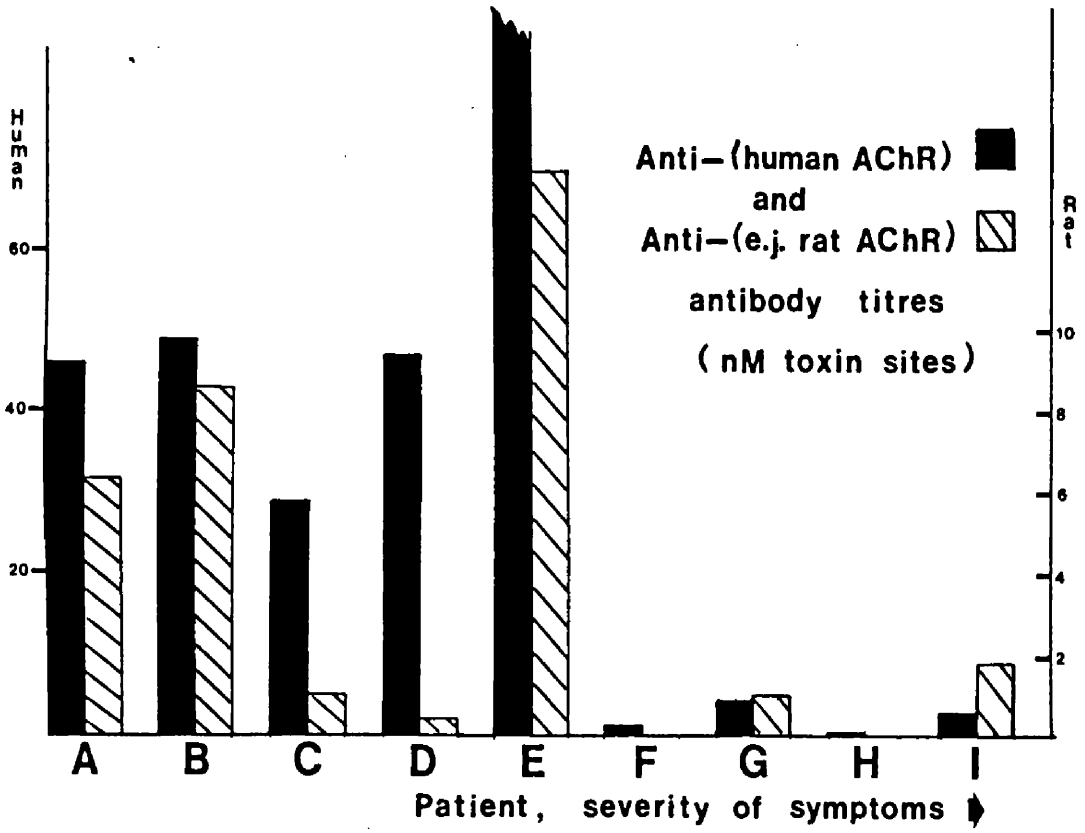


FIGURE 2. Anti-(human AChR) and anti-(extrajunctional rat AChR) antibody titers of serum samples from nine different myasthenic patients. Titers are expressed as nM α-bungarotoxin binding sites.

prehensive study in which serum samples from a selected group of myasthenic patients were systematically analyzed for both anti-(extrajunctional rat AChR) and anti-(human AChR) antibodies over an extended period of time. In order that the study might extend over periods during which the patients experienced both a range of clinical states and widely varying anti-(human AChR) antibody titers, partients were selected so that each underwent at least one series of plasma-exchange during the course of the investigation.[9]

The overall results of the investigation are shown in FIGURE 3. The ratio of anti-(extrajunctional rat AChR) to anti-(human AChR) antibody titer was found to vary considerably between the thirteen patients examined, ranging from 0.01 to 0.63. Nevertheless, the ratio for a given patient was found to remain remarkably constant over periods of up to 36 months (FIGURE 3) despite the fact that in all cases the anti-(human AChR) antibody levels fluctuated widely during the period of investigation, particularly during plasma-exchange and subsequent periods of immunosuppression.[9] The detailed behavior of antibody titers is typified by the patterns shown in FIGURE 4 for patient 12 (FIGURE 3). This 64-year-old male patient underwent two courses of plasma exchange coupled with immunosuppressive therapy during the study and his clinical response to these treatments has been described in detail by Behan *et al.*[10] At the beginning of the present investigation, the patient was classified as having a severe, generalized form of the disease. He was subjected

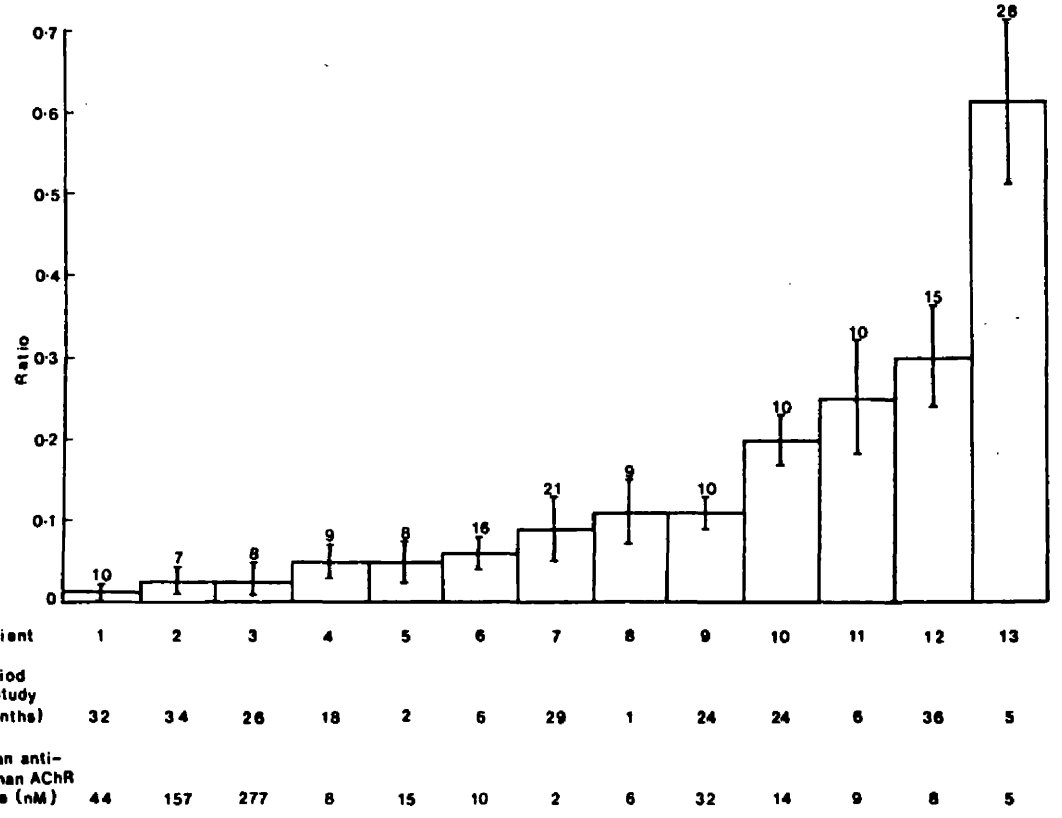


FIGURE 3. Ratios of anti-(extrajunctional rat AChR) antibodies: anti-(human AChR) antibodies for repeat serum samples from thirteen different myasthenic patients. The heights of the columns represent mean ratios. ± Standard deviations are shown by the error bars surmounting the columns and the number of samples assayed is shown above each column. Mean anti-(human AChR) antibody titers shown below the histograms are expressed as nM α-bungarotoxin binding sites.

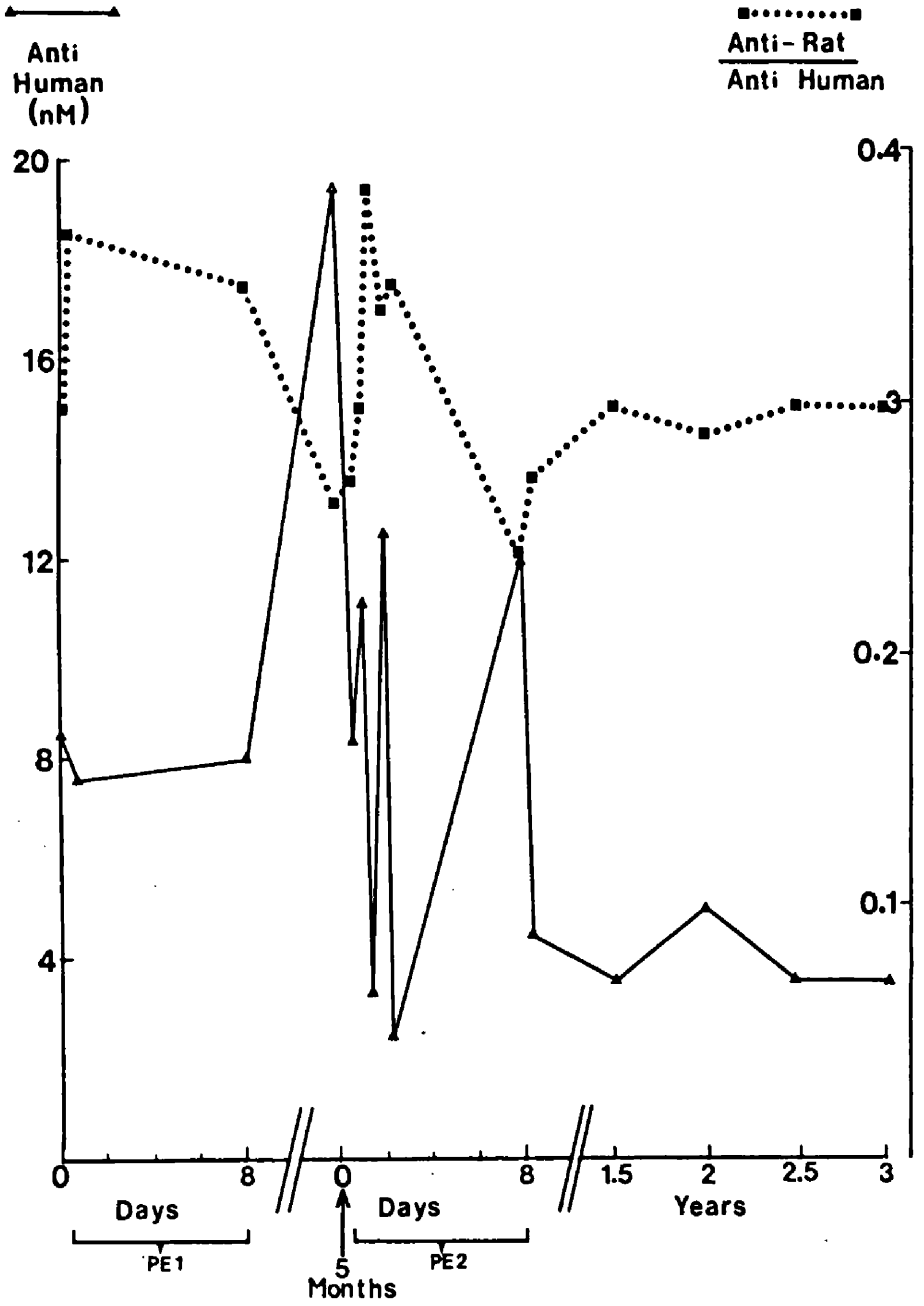


FIGURE 4. Ratios of anti-(extrajunctional rat AChR) antibodies: anti-(human AChR) antibodies and anti-(human AChR) antibody titers for patient 12 (FIGURE 3) over a 36-month period. Titers are expressed as nM α-bungarotoxin binding sites.

to a course of six plasma exchanges over an eight-day period (PE 1 FIGURE 4) at the end of which he showed little clinical improvement or overall change in anti-(human AChR) antibody titer. Five months later his condition worsened dramatically and his anti-(human AChR) antibody titer rose sharply.[9] Through a second course of five plasma exchanges (PE 2 FIGURE 4) his antibody titer fell in a familiar zig-zag pattern to a lower level and the patient showed marked clinical improvement. Immunosuppression was maintained over a three month period which resulted in further improvement in the patient. This improvement has now been sustained for two and a half years and

corresponds to a lowered and stabilized anti-(human AChR) antibody titer (FIGURE 4). Throughout these fluctuations in antibody titer and clinical condition, the ratio of anti-(extrajunctional rat AChR) to anti-(human AChR) antibody has varied little about a mean value of approximately 0.3. It is tempting to see some importance in the sharp fall in ratio coincident with the myasthenic crisis occurring after five months and the subsequent rise in ratio corresponding to the clinical improvement accompanying plasma exchange. However the inverse relationship between anti-(human AChR) antibody titer and the anti-(rat AChR) antibody to anti-(human AChR) antibody ratio apparent in FIGURE 4 was not always observed in other patients and discussion of the significance of these variations must await further data.

A further detailed example is shown in the case of patient 9, a thirty-year-old woman who was classified as Osserman Grade 3 [11] initially and following an eleven day course of eight plasma exchanges, coupled with immunosuppression,[10] improved to Grade 0, a state which has been maintained for two years. In parallel with this clinical improvement the anti-(human AChR) antibody titers fell and stabilized at a lower value whereas the ratio of anti-(extrajunctional rat AChR) antibodies to anti-(human AChR) antibodies remained remarkably constant at approximately 0.1 throughout these changes (FIGURE 5). Comparison of the data for patient 12 (FIGURE 4) with corresponding values for patient 9 (FIGURE 5) suggests the possibility of an inverse relationship between anti-(human AChR) antibody titer and the anti-(rat AChR): anti-(human AChR) antibody ratio for different patients. An examination of the data shown in FIGURE 3, however, shows that while there may be a general tendency for low anti-(rat AChR)—anti-(human AChR) ratios to be associated with high mean anti-(human AChR) antibody titers—there is no clear inverse correlation between the two values across the range of patients studied.

In addition to the anti-(extrajunctional rat AChR) antibodies, the anti-(junctional rat AChR) antibodies were also systematically assayed in the sera of the same thirteen myasthenia patients. In agreement with Weinberg and Hall [12] we find that the titers obtained by using junctional rat AChR as antigen in the radioimmunoassay are almost always lower than those obtained by using extrajunctional rat AChR. In our study the ratio of anti-(junctional rat AChR) antibodies to anti-(extrajunctional rat AChR) antibodies was found to vary from 0.0 to 1.0 depending on the patient but to be relatively constant for a given patient (FIGURE 6) over periods of up to 34 months which, as discussed above, included wide variations in both clinical state and anti-(human AChR) antibody titer. Weinberg and Hall [12] reported data from ten samples of myasthenic sera and found ratios of anti-(junctional rat AChR) antibodies to anti-(extrajunctional rat AChR) antibodies ranging from 0.6 to 1.0. They suggested that the apparent selectivity of some myasthenic antibodies for extrajunctional receptors might be made use of in the detection and purification of such receptors. The feasibility of this approach is supported and extended by our results which show that particular patients (e.g., patient 2, FIGURE 6) can have sera with relatively high anti-(extrajunctional rat AChR) antibody titer and yet no detectable antibodies to junctional rat receptor.

The relatively constant proportions of both anti-(junctional rat AChR) antibodies and anti-(extrajunctional rat AChR) antibodies to anti-(human AChR) antibodies within an individual patient over extended periods suggests that the overall pattern of anti-AChR antibody subpopulations is specific for that patient. It is also apparent from our results that the pattern can vary

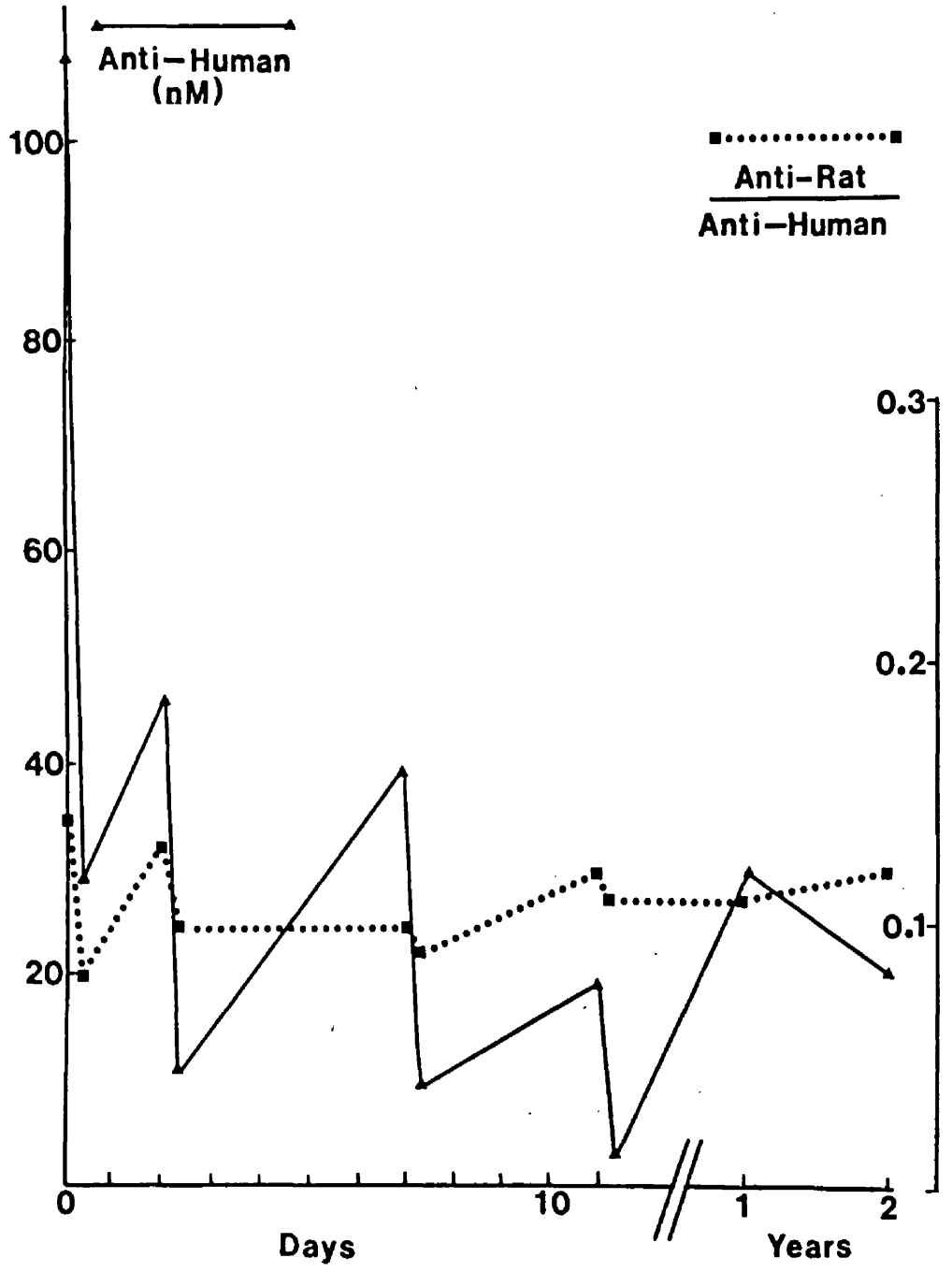
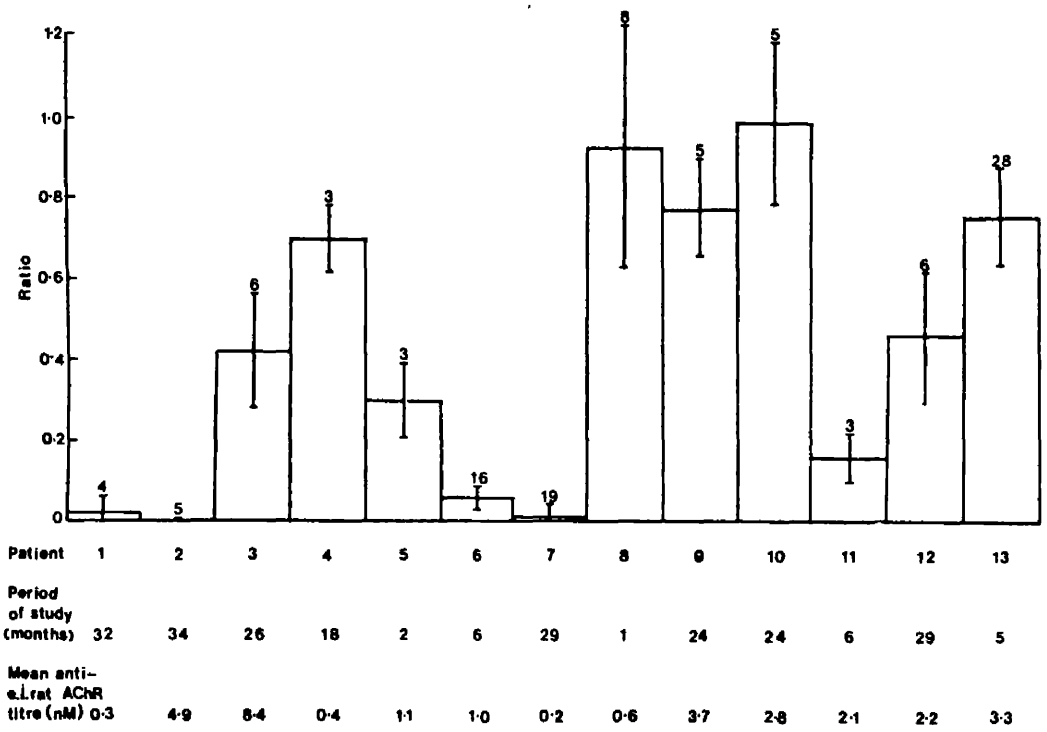


FIGURE 5. Ratios of anti-(extrajunctional rat AChR) antibodies: anti-(human AChR) antibodies and anti-(human AChR) antibody titers for patient 9 (FIGURE 3) over a 24-month period. Titers are expressed as nM α-bungarotoxin binding sites.



Patient	1	2	3	4	5	6	7	8	9	10	11	12	13
Period of study (months)	32	34	26	18	2	6	29	1	24	24	6	29	5
Mean anti-e.l.rat AChR titre (nM)	0·3	4·9	8·4	0·4	1·1	1·0	0·2	0·6	3·7	2·8	2·1	2·2	3·3

FIGURE 6. Ratios of anti-(junctional rat AChR) antibodies: anti-(extrajunctional rat AChR) antibodies for repeat samples from the myasthenic patients shown in FIGURE 3. The heights of the columns represent mean ratios. ± Standard deviations are shown by the error bars surmounting the columns and the number of samples assayed is shown above each column. Mean anti-(extrajunctional rat AChR) antibody titers shown below the histograms are expressed as nM α-bungarotoxin binding sites.

widely between patients. Complementary but more ambiguous evidence for the same phenomenon is provided by a different set of experiments with sera taken from ten of our thirteen (FIGURES 5 and 6) myasthenic patients over periods of several months which, as before, included at least one plasma exchange with associated changes in clinical state and anti-(human AChR) antibody titer. Excess of each sample of myasthenic serum was used, in combination with goat anti-(human IgG) antiserum to precipitate ^{125}I-α- bungarotoxin-labelled AChR from a human muscle extract. FIGURE 7 shows the results of these experiments in which the amount of labelled AChR precipitated is expressed as a percentage of that precipitated by sera from patient 3, an amount arbitrarily defined as 100%. The amount of ^{125}I-α-bungarotoxin-labelled AChR precipitated varies between patients from 48% to 100% but is essentially constant for a given patient over the period of study (FIGURE 7). These findings might result from the absence of particular anti-AChR antibody subpopulations in the sera from certain myasthenic patients. This explanation would also require heterogeneity in the toxin-binding components of the muscle extract which could well reflect the presence of proteolysis-induced fragments in the crude detergent solution. A second possible explanation involves the presence in different myasthenic sera of different concentrations of antibodies directed at the α-bungarotoxin binding site of the receptor molecule. Such antibodies could displace ^{125}I-labelled toxin from the toxin-AChR complex, so diminishing the amount of labelled AChR precipitated by the relevant antiserum sample.

Preliminary experiments carried out by us favor the former explanation for partial precipitation of ^{125}I-α-bungarotoxin-labelled AChR by excess of certain myasthenic sera in that incomplete precipitation of labelled AChR by excess of certain myasthenic sera can be increased to that characteristic of serum from patient 3 by addition of the latter serum. This question is being further investigated in our laboratory.

Whether either one or both of the above explanations is valid, the constant value for a given patient of the percentage precipitation of labelled AChR shown in FIGURE 7 provides further evidence for a patient-specific pattern of anti-AChR antibody subpopulations in myasthenia gravis. The unchanging nature of this antibody pattern suggests that the pattern is established in all cases before the start of our investigations and would be consistent with an initial limited exposure of the AChR antigen to the immune system. The data argue against the production of autoantibodies to AChR as a response to a continuous presentation of antigen that might result, for example, from the shedding of receptor from the muscle surface or its exposure in modified form at that site. In this case the immune response would be secondary to some primary pathogenic factor and the antibody pattern would be expected to vary with each representation of antigen.

Examination of the case histories of this group of patients from the time of our initial results[13] has not revealed any interesting relationship between antibody subpopulation patterns and a particular clinical history. However, it can be hoped that more extensive investigations of the detailed nature of the

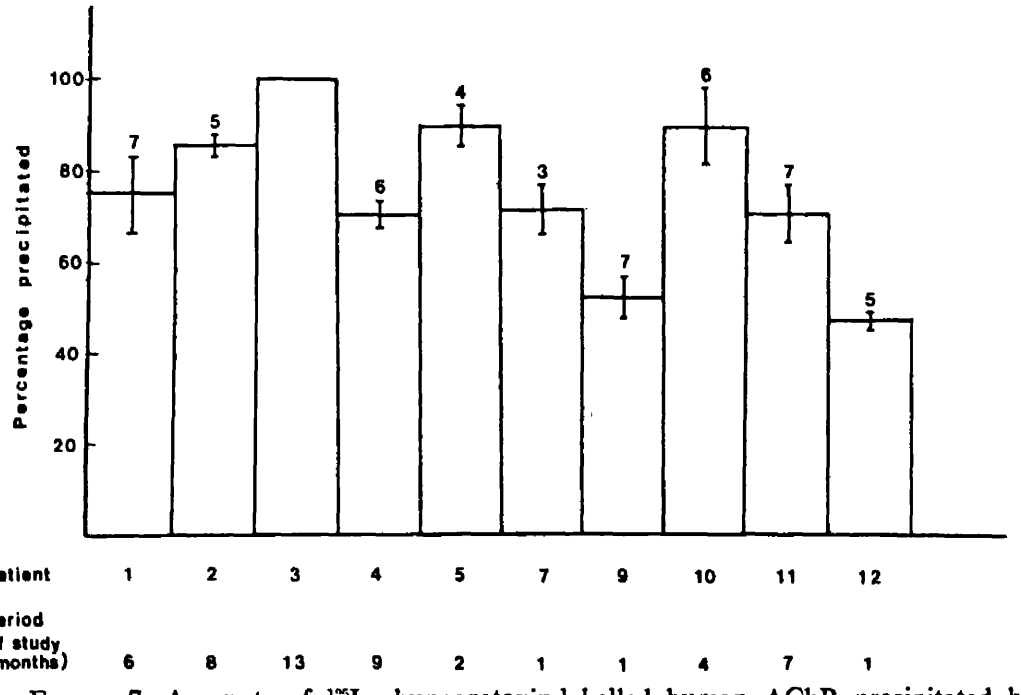


FIGURE 7. Amounts of ^{125}I-α-bungarotoxin-labelled human AChR precipitated by repeat serum samples from ten of the patients shown in FIGURE 3. Excess of the myasthenic serum was used in combination with goat anti-(human IgG) antiserum to precipitate labelled AChR and values are expressed as percentages of the amount of labelled AChR precipitated by serum from patient 3 under identical circumstances. The heights of the columns represent mean percentages. ± Standard deviations are shown by the error bars surmounting the columns and the number of samples assayed is shown above each column.

anti-AChR antibody subpopulation patterns in myasthenic sera may shed some light not only on the aetiology of the disease but also on its particular progress in individual patients.

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Discussion

A. Vincent: We have a series of patients whose serums were unable to fully precipitate the receptor. This appeared to be due to a subpopulation of antibodies that are capable of displacing the toxin off the receptor. Have you looked at this phenomenon?

R. Harrison: We have not. We assumed that failure to obtain complete precipitation was due either to the absence of subpopulations of antibody that bind toxin, or to proteolysis of receptor in the assay.

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An assessment of radioimmunoassay procedures for determination of anti-acetylcholine receptor antibodies in the sera of patients with myasthenia gravis

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SUMMARY A reproducible radioimmunoassay procedure for the determination of anti-acetylcholine receptor antibodies in the sera of patients with myasthenia gravis is described and examined in detail. The assay combines features of a number of methods previously outlined and allows repeat determinations of antibody titre in a given myasthenic serum sample with coefficient of variation 6%. The mean ± standard deviation for normal human serum anti-acetylcholine receptor antibodies was found by this procedure to be 0·024 ± 0·033 nmol/l α-bungarotoxin binding sites whereas the range for myasthenic patients was 0–139·14 nmol/l with a mean value of 7·55 nmol/l α-bungarotoxin binding sites.

Myasthenia gravis is generally recognised to be an autoimmune disease in which patients develop an immune response to self nicotinic acetylcholine receptor (AChR) at the neuromuscular junction.[1] Approximately 90% of myasthenia patients show elevated serum levels of anti-AChR antibodies,[2] and there is an increasing demand for determination of such levels as an aid to diagnosis. Despite the usual presence of anti-AChR antibodies in the sera of myasthenia gravis patients, correlation of antibody titre with severity of symptoms is generally poor between patients.[3–5] Nevertheless, titres followed for a given patient over a period of time can reflect the clinical state of the patient, and serial assays can be used to monitor the effects of particular treatment regimens.[6]

A basic radioimmunoassay for the determination of anti-AChR antibody titres in human sera has been reported by a number of groups of workers.[3 7–10] The assay involves specific labelling of human AChR with radiolabelled snake venom α-toxin followed by the use of the labelled AChR, in excess, to precipitate anti-AChR antibodies present in the patient's serum. The assay is relatively complex, and a number of aspects of the published procedures differ widely, depending upon the authors. In many cases, moreover, exact experimental details are unclear, and the reasons underlying the choice of a particular technique or set of conditions are unspecified. We now report an examination of a number of variable factors in the radioimmunoassay, the results of which lead to an overall method suitable for routine application.

Material and methods

Myasthenic and normal human sera were quickly frozen and stored at −20°C before assay. Human muscle was obtained from lower limb amputations. Within 15 minutes of amputation, calf muscle was crudely dissected free from fat, tendon, and skin, transported in ice or solid carbon dioxide (approx. 20 minutes), and stored at −80°C.

α-Bungarotoxin from *Bungarus multicinctus* was purchased from Boehringer, Mannheim, West Germany as a lyophilised powder (1 mg), which was dissolved in 0·05 mol/l potassium phosphate buffer, pH 7·5 (2 ml), and stored at −20°C before use. Na ^{125}I in dilute NaOH solution (100 mCi/ml) was supplied by the Radiochemical Centre, Amersham, UK, and benzoquinonium chloride was a generous gift from Stirling Winthrop Inc, Rensselaer, NY, USA. All other chemicals were from Sigma Chemical Co, Kingston upon Thames, UK, or from BDH Chemicals, Poole, Dorset, UK.

^{125}I-Labelled α-bungarotoxin was prepared

essentially according to the procedure of Urbaniak *et al.*[11] as follows. Stock solutions (20 μl) of α-bungarotoxin and Na ^{125}I (10 μl) were mixed with 0·25% (w/v) chloramine T in 0·05 mol/l potassium phosphate buffer, pH 7·5 (20 μl), and stirred for 1 minute at 22°C. 0·016% (w/v) Sodium metabisulphite in 0·05 mol/l potassium phosphate buffer, pH 7·5 (750 μl), and 1% (w/v) KI in the same buffer (200 μl) were added to the iodination mixture to stop the reaction, giving a final total volume of 1 ml. ^{125}-I Labelled α-bungarotoxin was separated from free ^{125}I by passage of the reaction mixture down a column (27 cm × 1 cm) of Sephadex G–25, which had been equilibrated with 0·01 mol/l potassium phosphate buffer, pH 7·5, containing 1% (w/v) bovine serum albumin. The column was eluted with the same buffer. Fractions (1 ml) were collected, and samples (10 μl) were counted for radioactivity. Fractions corresponding to the first protein-containing peak of radioactivity were combined to give approximately 0·4 μmol/l ^{125}I-labelled α-bungarotoxin with specific activity approximately 0·3 mCi/ml. The labelled toxin was stored at 4°C for up to three weeks.

Alternatively, ^{125}I-labelled α-bungarotoxin is available from the Radiochemical Centre, Amersham, UK.

Goat anti-(human IgG) antiserum was obtained by repeated (three-weekly) intramuscular injections of purified[12] human IgG [1·5 mg in Freund's adjuvant (0·5 ml) ('complete' for the first injection and 'incomplete' thereafter)]. After all injections but the first, serum (200 ml) was taken, and the presence of anti-(human IgG) antibodies was established by double-diffusion against human IgG.[13] The presence of adequate antibody titres was checked by use directly in the radioimmunoassay (see Results and Discussion section) of a standard sample of myasthenic serum when maximum titres should be obtained by incubation of 100–200 μl goat antiserum. Suitable titres were normally achieved after the third injection.

Goat anti-(human IgG) antiserum of suitable titre was alternatively purchased from Miles Laboratories, Stoke Poges, Bucks, UK.

^{125}I-Radioactivity was routinely counted in an LKB 1280 Ultrogamma counter.

EXTRACTION OF HUMAN AChR

Frozen human muscle (approx. 200 g) was chopped finely and homogenised in a Sorvall Omnimix at maximum speed for 1 minute at 4°C in 0·02 mol/l potassium phosphate buffer, pH 7·5 (4 volumes), containing 1 mmol/l EDTA, 0·5 mmol/l phenylmethylsulphonyl fluoride, 0·1 mmol/l benzethonium chloride, and 0·01% (w/v) NaN_3 and centrifuged at 2000 *g* for 45 minutes at 4°C. The pellet was resuspended and homogenised as before in the above buffer (1 volume) containing additionally 2·5% (v/v) Triton X–100 (extraction buffer), stirred for 16 hours at 4°C (or for 3 hours at 20°C), and centrifuged at 20 000 *g* for 5 minutes at 4°C followed by 100 000 *g* for 45 minutes at 4°C. The resulting supernatant was filtered through glass wool and stored at 4°C.

Re-extraction of the muscle homogenate was occasionally found to be profitable in the case of preparations with relatively high activity. Thus the pellet obtained from centrifugation at 100 000 *g* was rehomogenised in extraction buffer (1 volume) as described above, stirred for 16 hours at 4°C, and centrifuged as above to give a second supernatant with α-toxin-binding activity.

DETERMINATION OF AChR CONCENTRATION

The concentration of AChR in soluble extracts of human muscle was determined by the specific binding of ^{125}I-labelled α-bungarotoxin. The receptor extract (100 μl) was incubated with 15 nmol/l ^{125}I-labelled α-bungarotoxin solution in extraction buffer (50 μl) for 15 minutes at 22°C before addition of saturated $(NH_4)_2\ SO_4$ solution to give 40% saturation overall and further incubation for 24 hours at 4°C. The resulting precipitate was collected by vacuum filtration in a Millipore filter tower on Whatman GFC glass fibre filters (0·5 cm), washed with 40% saturated aqueous $(NH_4)_2SO_4$ solution, and counted for radioactivity. Sufficient excess of α-toxin was ensured by repetition of the assay using serial two-fold dilutions of receptor extract in extraction buffer (100 μl) when a linear relationship between dilution number and precipitated radioactivity should be obtained.

Specific binding of α-toxin was blocked in parallel incubations performed as above but containing additionally 1·0 nmol/l benzoquinonium chloride or 0·1 mmol/l d-tubocurarine. Subtraction of the counts so obtained gave specifically-bound radioactivity in the test sample.

ASSAY OF ANTI-AChR ANTIBODIES

Detergent extract (5 ml) of human muscle containing approximately 0·5 nmol/l specific toxin-binding sites (see below) was incubated with a 10-fold molar excess of ^{125}I-labelled α-bungarotoxin in extraction buffer (<100 μl) for 15 minutes at 22°C. A sample (100 μl) of the resulting solution was incubated with myasthenic serum (5 μl) for 16 hours at 4°C (or for 2 hours at 22°C), and the labelled AChR-antibody complex was precipitated by addition of goat anti-(human IgG) antiserum (100–200 μl, see Methods section) and storage for 24 hours at 4°C (or for 2

hours at 22°C). The resulting precipitate was separated by centrifugation at 3000 *g* for 10 minutes, and the pellet was washed twice with 0·01 mol/l potassium phosphate buffer, pH 7·5, containing 0·15 mol/l NaCl, 1% (v/v) Triton X-100, and 0·1% (w/v) NaN_3 and counted for radioactivity. Specific binding of α-toxin was blocked in parallel incubations performed as above but containing additionally 1·0 mmol/l benzoquinonium chloride (or 0·1 mmol/l d-tubocurarine or 0·1 mmol/l non-radioactive bungarotoxin). Subtraction of the counts so obtained gave specifically-bound radioactivity in the test sample, which can then be related to the concentration of α-bungarotoxin-binding sites in the AChR extract. Thus the antibody titre is expressed as moles of specific α-bungarotoxin binding sites precipitated per litre of serum.

Maximal formation of AChR-antibody complexes was routinely checked by repeating the assay using serial twofold dilutions (with normal human sera) of antisera in order to ensure a linear relation between the volume of undiluted serum and precipitated radioactivity.

Results and discussion

The concentration of soluble AChR in the primary detergent extracts of human muscle was found to vary from 0·1 to 2·5 nmol/l α-bungarotoxin-binding sites over 20 preparations with a mean value of 0·52 nmol/l toxin-binding sites. This represents a mean recovery of 0·49 pmol toxin-binding sites per gram muscle.

The wide variation in α-bungarotoxin-binding activities of different muscle samples may reflect differences in the clinical state of the muscle samples before amputation. Muscle was always obtained from legs amputated because of either vascular disorders or diabetic gangrene, and it is possible that the different samples had been subjected to varying periods of ischaemia with attendant partial autolysis. Moreover most amputees were patients over 60 years of age who are known[14] to be subject to significant and varying degrees of motor denervation. This raises the further possibility of variable extents of acetylcholine receptor proliferation[15] in the muscle samples we examined, although this was not reflected in binding studies carried out by us on purified receptor preparations.[16]

Attempts to prepare useful extracts of acetylcholine receptor from postmortem muscle proved to be generally unsatisfactory, giving receptor concentrations of less than 0·1 nmol/l toxin binding sites. This probably reflects the occurrence of autolysis during the extended periods (more than 6 hours) elapsing between death and freezing of the muscle.

With regard to the method of extraction of AChR, the inclusion in the buffers of phenylmethylsulphonyl fluoride and benzethonium chloride as anti-proteolytic agents was found generally to improve the yield of AChR. Similar yields of AChR were found whether extraction in Triton-containing buffer was carried out for 16 hours at 4°C or for 3 hours at 20°C.

The concentration of AChR extract (0·5 nmol/l) recommended in the Methods section for use in the radioimmunoassay of anti-AChR antibody titres is only slightly less than the mean value in the extracts. This inevitably means that many preparations contain concentrations of receptor suboptimal for this purpose, and re-extraction of the muscle homogenate (Methods section) was found to give secondary extracts with $46 \pm 33\%$ ($\pm$ SD, 6 preparations) of the toxin-binding activity of the primary extract. Such re-extraction was often worthwhile in the case of relatively high-activity receptor preparations in order to supplement stocks of useful receptor.

Storage of AChR extracts at 4°C for periods of up to three months led to less than 40% loss of toxin-binding activity.

In the determination of AChR concentration, pre-incubation of AChR with ^{125}I-labelled α-bungarotoxin for periods of longer than 15 minutes, up to 5 hours, was found not to affect the results. On the other hand, incubation of the AChR-toxin complex with $(NH_4)_2SO_4$ for less than 16 hours led to diminished precipitation of radioactivity.

Separation of AChR-toxin complex from free toxin can be effected alternatively by filtration through DEAE cellulose filter discs on a Millipore filtration system[10] followed by washing the discs with 0·01 mol/l potassium phosphate buffer, pH 7·5, containing 0·1% (w/v) bovine serum albumin, 0·01% (w/v) NaN_3, and 1·0% (v/v) Triton X-100. In our hands, specifically-bound radioactive counts were consistently found to be 60–85% lower than those obtained by using $(NH_4)_2SO_4$ precipitation, a decrease that is at least partially attributable to saturation of the filters by the high levels of protein in the extract.[9,17] These levels can be reduced by dilution of the extract before filtration, which can lead, however, to unacceptable loss of sensitivity in the case of lower activity extracts. The DEAE filter assay is, nevertheless, much faster than that based on $(NH_4)_2SO_4$ precipitation and can be used to give rapid estimates of AChR concentration for use in the radioimmunoassay of anti-AChR antibodies.

A third method of separation of AChR-toxin complexes from free toxin in the routine assay of AChR concentrations involves the addition of excess myasthenic serum followed by goat

anti-human γ-globulin serum, as described by Lindstrom.[8] Varying results can, however, be obtained by using sera from different patients in this assay, and care must be taken to use serum from a selected individual if values given by this method are to be consistent and comparable with those obtained by other means. In a study of serum samples taken from 10 myasthenic patients over a period of several months, we found that amounts of AChR-toxin complex precipitated from a standard extract of AChR by excess of myasthenic serum varied between serum samples but was constant for sera from a given patient.[18] The greatest precipitation of complex was consistently effected by sera from one particular patient and represented 80–90% of that brought down by $(NH_4)_2SO_4$. Sera from the remaining nine patients precipitated 46–86% of the highest value.

Identical results in the assay for AChR were obtained when either 1·0 mmol/l benzoquinonium chloride or 0·1 mmol/l d-tubocurarine were used to block specific binding of ^{125}I-labelled α-bungarotoxin to the receptor. We found that lower concentrations of the respective blocking agents led to decreased inhibition of toxin binding. Closely similar results were also obtained when specific binding was eliminated by treatment of the control samples with excess (in this case 0·1 mmol/l) of unlabelled α-bungarotoxin, as described by Newsom-Davis *et al.*[10] The use of a large excess of unlabelled ligand in this way is a commonly used technique in studies of receptor binding. The method depends upon the displacement of labelled ligand only from sites having high affinity in the context of the assay and as such can be subject to interference from 'non-specific' binding with high affinity. Such interference could also affect assays for specific binding that depend upon displacement of radio-labelled ligand by a chemically different ligand (eg, by benzoquinonium or d-tubocurarine). In this case, however, the probability of competition between labelled and unlabelled ligand for the common 'non-specific' site is much less, and, in general, the use of well-characterised, structurally distinct unlabelled ligands is to be preferred in the displacement of specific binding.[19]

The concentration of ^{125}I-labelled α-bungarotoxin (15 nmol/l) recommended in the assay for AChR activity represents 5 nmol/l in the incubation mixture, which is approximately 14 times the corresponding concentration of AChR obtained by using an average receptor preparation. Figure 1 shows that precipitation of AChR-toxin complex can continue to increase as the toxin: AChR molar ratio increases up to values of 6–10. In practice, it was found that use of the above concentrations of ^{125}I-labelled

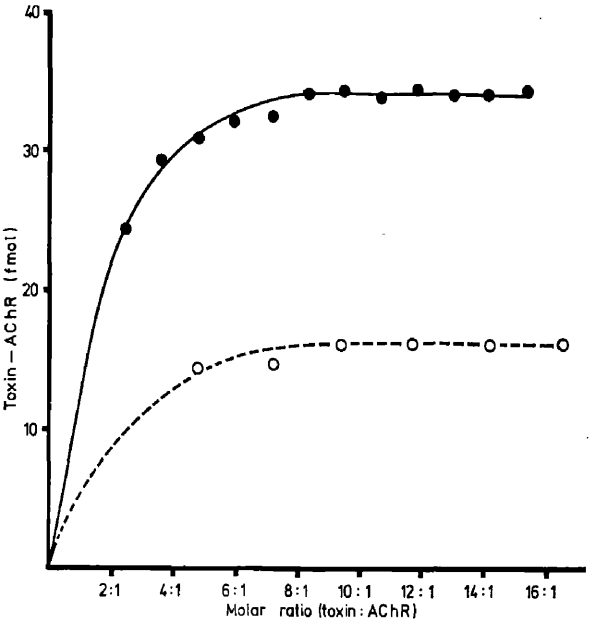

Fig. 1 *Formation of toxin-AChR complex at varying molar ratios of ^{125}I-labelled α-bungarotoxin to AChR. Each of two samples of AChR extract (100 μl) was separately incubated with increasing concentrations of ^{125}I-labelled α-bungarotoxin in extraction buffer (50 μl) for 15 minutes at 22°C. $(NH_4)_2SO_4$ was then added to 40% saturation, and the mixture was further incubated for 24 hours at 4°C. The precipitates were collected on glass fibre filters, washed with 40% saturated $(NH_4)_2SO_4$ solution, and counted. Specifically-bound α-toxin was determined by using benzoquinonium chloride in parallel incubations as described in the text.*

α-bungarotoxin rarely led to non-linear response on dilution of AChR in the assay (see the Methods section). The dissociation constant, K_D, for binding of ^{125}I-labelled α-bungarotoxin to AChR determined from the data of Fig. 1 had a value of 0·32 nmol/l, a value some 15 times lower than the concentration of α-toxin used in the assay.

In the assay for anti-AChR antibodies, incubation of labelled toxin-AChR complex with myasthenic serum for varying lengths of time at 4°C showed that apparent anti-AChR antibody titres increased up to approximately 16 hours, after which titres remained constant for incubation times up to a total of 48 hours. Identical titres were obtained for the same myasthenic serum samples when incubations were alternatively carried out for 2 hours at 22°C.

Incubation of labelled AChR-antibody complexes with goat anti-(human IgG) antisera at 4°C was found to lead to maximal anti-AChR antibody titres only after 24 hours. Titres fell slightly when longer incubation times, up to 60 hours, were employed. Results identical with those obtained by incubation for 24 hours at 4°C were obtained

alternatively by incubation for 1, 2, or 4 hours at 22°C.

As described in the Methods section, serial dilutions of myasthenic sera were made routinely in order to ensure that the antibody assay was being done with sufficient molar excess of labelled AChR. An examination of the dependence of apparent antibody titres on the molar ratio of labelled AChR to specific antibodies showed that molar ratios of approximately 3:1 were sufficient to ensure maximal precipitation of anti-AChR antibodies in the myasthenic samples tested (Fig. 2). Maximal formation of AChR-anti-AChR antibody complexes requires not only a minimum AChR-antibody ratio but also a minimum concentration of AChR, which in turn depends upon the dissociation constants of the AChR-antibody complexes. A study of three myasthenic sera showed (Fig. 3) that minimal concentrations of AChR of 0·3–0·5 nmol/l, depending on the serum sample, were sufficient to effect maximum formation of complex. This range is just below the level 0·5 nmol/l of AChR concentration suggested for use in the radioimmunoassay. Nevertheless the results indicate that the latter value may be close to a minimum acceptable figure for some myasthenic sera and that higher concentrations of receptor, if they can be obtained, may well be used to advantage in the radioimmunoassay procedure.

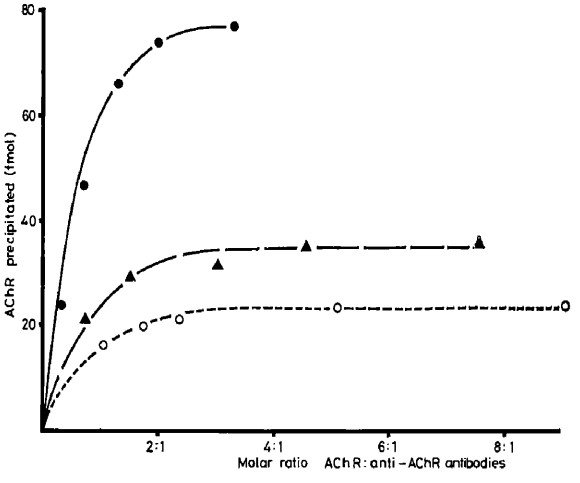

Fig. 2 *Formation of AChR-anti-AChR antibody complexes at varying molar ratios of toxin-labelled AChR to antibody. A single appropriate dilution of serum was made from each of three different myasthenic patients. Samples (5 μl) of each were incubated with increasing amounts of a fixed concentration of toxin-labelled AChR, and labelled AChR-antibody complexes were precipitated and counted as described for the antibody assay procedure (see text). The molar concentrations of antibody were, in each case, obtained from the maximum amount of AChR precipitated. Concentrations of toxin-labelled AChR were ●, 1·02 nmol/l, ▲, 1·32 nmol/l, and ○, 0·32 nmol/l.*

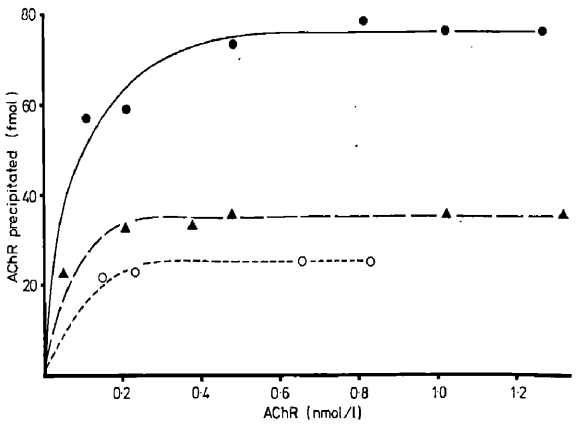

Fig. 3 *Formation of AChR-anti-AChR antibody complexes by varying concentrations of toxin-labelled AChR. A single appropriate dilution of serum was made from each of three different myasthenic patients. Samples (5 μl) of each were incubated with a constant amount of toxin-labelled AChR at different concentrations so that a constant molar ratio of toxin-labelled AChR: anti-AChR antibodies was maintained. These ratios were ●, 3:1; ▲, 7:1; ○, 10:1. Labelled AChR-antibody complexes were precipitated and counted as described for the antibody assay procedure (see text).*

The procedure for radioimmunoassay of anti-AChR antibodies outlined in the Methods section allows the determination of relatively reproducible titres. Thus, repeat determinations on an individual serum sample using a single extract of acetylcholine receptor and a single freshly prepared sample of ^{125}I-labelled α-bungarotoxin gave values of 2·99 ± 0·17 (± SD, 15 assays) nmol/l α-bungarotoxin binding sites. Repeat assays of the same serum using a single extract of acetylcholine receptor and four different preparations of ^{125}I-labelled α-bungarotoxin gave values of 3·23 ± 0·31 (± SD) nmol/l α-bungarotoxin binding sites (where the mean value for each toxin preparation resulted from 15 separate assays). Repeat assays of a different serum sample using a single preparation of ^{125}I-labelled α-bungarotoxin and four different extracts of acetylcholine receptor gave values of 1·80 ± 0·22 (± SD) nmol/l α-bungarotoxin binding sites (where the mean value for each extract of acetylcholine receptor resulted from 10 separate assays).

It can be seen that rather more variability in these assays is usually found when different ^{125}I-labelled α-bungarotoxin and acetylcholine receptor preparations are used. Titres obtained for a given serum sample using a single extract of acetylcholine receptor and sample of labelled toxin change little over a period of less than three weeks. Thus repeat assays of a single serum sample using one extract of acetylcholine receptor made using freshly ^{125}I-labelled α-bungarotoxin and the same toxin after

storage at 4°C for two and a half weeks gave values of 2·89 ± 0·10 (± SD, 15 assays) and 2·99 ± 0·17 (± SD, 15 assays) nmol/l α-bungarotoxin binding sites respectively. Storage of labelled toxin for periods of longer than three weeks was found to lead to much greater variation in antibody titres, and ^{125}I-toxin was not used beyond this point. We have found no evidence of systematic variation in antibody titres of given serum samples arising from changes in the extracts of acetylcholine receptor on storage at 4°C for up to three months.

In view of the somewhat greater consistency of assays obtained by using single ^{125}I-labelled α-toxin and receptor preparations, there is some advantage to be gained by storing serial serum samples taken from an individual patient over a period of treatment in order to assess most accurately the effect of such treatment on anti-AChR antibody titres. Changes in titre induced by treatments such as immunosuppression and plasma-exchange[6 20] are, however, commonly much greater than the variations observed using several preparations of labelled toxin and receptor, and the precaution is usually not essential.

We have found that it is useful to store (at −80°C) aliquoted standard samples of myasthenic serum which can be used to check the performance of each new acetylcholine receptor extract and the ^{125}I-labelled α-toxin preparation in the radioimmunoassay.

Apart from the use of the radioimmunoassay in monitoring a patient's progress, the assay has, as already mentioned, a limited use in diagnosis and while not all clinically diagnosed myasthenic patients have anti-AChR antibody titres significantly above the normal range, most do. Assay of normal volunteers by the procedure discussed here gave values of 0·024 ± 0·033 (± SD, duplicate assays on 20 individuals) nmol/l α-bungarotoxin binding sites, while anti-AChR antibody titres in clinically diagnosed myasthenic patients ranged from 0 to 139·14 nmol/l α-bungarotoxin binding sites with a mean value of 7·55 nmol/l sites.

Perhaps the most troublesome aspect of the assay procedure is the requirement for a minimum concentration of acetylcholine receptor in the extracts of human muscle, by no means all of which are directly usable in the radioimmunoassay. It is possible to increase the concentration of receptor extracts by vacuum dialysis which, although it gave increased non-specific binding of α-bungarotoxin and corresponding loss of sensitivity in the assay, did not obviously affect the titres obtained. As has been discussed, it is difficult to predict which samples of amputated muscle will have high receptor activity, and the more readily obtained postmortem muscle usually gives very low activity extracts. It has been reported[21] that the use of AChR purified from rat muscle as antigen in the radioimmunoassay for anti-AChR antibodies in human sera gave the same titres as were obtained when purified human muscle AChR was used as antigen. In our experience, however, the use of rat receptor leads to very much lower titres compared with human AChR and consequently to a less sensitive system. Moreover, we found that correlation of disease severity with anti-AChR antibody titre was not improved by the use of rat antigen.[18 22] These results do not encourage the use of experimental animals as an alternative source of muscle receptor, and this conclusion is supported by the finding that the use of rabbit muscle AChR also leads to relatively low anti-AChR antibody titres in the radioimmunoassay.[17]

The temperatures and times of a number of the incubation steps in the overall procedures outlined here can be modified, as described, to fit the working convenience of laboratories engaged in the assay. Provided that the assay is performed within the limits discussed above, we have found the method to be reasonably convenient to apply and readily operated successfully by workers new to the determination.

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Interaction of Foetal and Adult Human Acetylcholine Receptors with Serum from Patients with Myasthenia Gravis

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Summary

Acetylcholine receptors were partially purified from foetal and adult human muscle and their binding to myasthenic serum was compared. No significant differences between the binding characteristics of the two receptor types were detected; indicating the absence, at least in 14–22-week-old foetuses of antigenic sites unique to foetal receptor and recognised by myasthenic sera.

Key words: *Acetylcholine receptors – Adult muscle – Foetal muscle – Myasthenia gravis*

Introduction

Sera from some 90% of myasthenic patients show the presence of anti-acetylcholine receptor (AChR) antibodies (Harrison and Behan 1983) as assayed by the commonly-used radioimmunoassay procedure (Lindstrom et al. 1976) using adult human AChR as antigen. If rat AChR is used as antigen in this assay then titres are generally smaller and are shown by a lower percentage of myasthenic patients (Savage-Marengo et al. 1979; McAdams and Roses 1980). Titres obtained by using extrajunctional AChR from denervated rats as antigen are usually found to be

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higher than by similar use of normal junctional rat AChR (Weinberg and Hall 1979; Savage-Marengo et al. 1980; Harrison et al. 1981), a fact which led Weinberg and Hall (1979) to suggest that embryonic AChR, in many ways similar to the extrajunctional adult receptor, might constitute the auto-immunogen in myasthenia gravis. In view of the fact that the use of rat AChR in this way can only give information about a limited, cross-reacting proportion of the total anti-human AChR antibody population, we have made a direct comparison of antibody titres in myasthenic patients by using, as antigen, adult human AChR on the one hand and foetal human AChR on the other.

Materials

Serum samples from myasthenic patients and normal human controls were quickly frozen and stored at −20°C. Adult human muscle was obtained from lower limbs amputated from patients with severe vascular disease and in 1 case from an otherwise healthy individual following a road traffic accident. Within 15 min of amputation, the calf muscles were crudely dissected free from fat, tendon and skin, transported in ice or solid carbon dioxide and stored at −80°C. Human foetuses were obtained from prostaglandin-induced terminations (foetal age 14–22 weeks); limb, back and intercostal muscles were dissected prior to use.

Goat anti-(human IgG) antisera were raised by repeated intramuscular injections of purified human IgG.

α-Bungarotoxin was obtained from the Boehringer Corporation (Lewes, Sussex, U.K.). *Naja naja siamensis* venom was from the Miami Serpentarium (Miami, FL, U.S.A.). Carrier-free Na ^{125}I in dilute NaOH solution (100 mCi/ml) was from the Radiochemical Centre, Amersham, Bucks, U.K. and benzoquinonium chloride was a generous gift from Stirling Winthrop Inc., Rensselaer, NY, U.S.A. Gel filtration reagents were supplied by Pharmacia Ltd., Hounslow, U.K. All other materials were from Sigma Chemical Co., Kingston-upon-Thames, U.K. or from B.D.H. Chemicals, Poole, Dorset, U.K.

Methods

^{125}I-labelled α-bungarotoxin and purified human AChR were prepared and the latter was assayed as previously described (Stephenson et al. 1981).

Anti-AChR antibody levels were determined essentially as described by Carter et al. (1981). Purified AChR (0–1.7 nM α-bungarotoxin-binding sites) was incubated with a 10-fold molar excess of [^{125}I]-α-bungarotoxin for 45 min at 22°C. Parallel control incubations contained additionally 1 mM benzoquinonium chloride to block specific binding of α-bungarotoxin. Duplicate samples (100 μl) of the resulting solutions were incubated with a fixed concentration of myasthenic serum or IgG (5 μl, appropriately diluted with normal human serum or IgG, respectively) for 2 h at 22°C (or 16 h at 4°C) and the labelled AChR–antibody complex was precipitated

by the addition of goat anti-(human IgG) antiserum (65 μl) and storage for 16 h at 4°C (or 2 h at 22°C). The resulting precipitate was separated by centrifugation at 3000 × *g* for 10 min (4°C) and the pellet was washed twice with 10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 1% (v/v) Triton X-100 and 0.1% (w/v) sodium azide and counted for radioactivity in an LKB 1280 Ultrogamma counter. Precipitated specific α-bungarotoxin binding sites were determined by subtraction of the counts obtained in the presence of benzoquinonium chloride. In each case excess of antigen was ensured by repeating the assay using serial 2-fold dilutions of serum or of IgG when a linear relationship should be obtained between the volume of undiluted serum and precipitated radioactivity.

Protein was determined by the method of Lowry et al. (1951) using BSA as standard.

Saturation curves of the form

$$y = \alpha\left(\frac{\gamma^x - 1}{\gamma^x + \beta}\right)$$

were fitted to data points by the Maximum Likelihood Method (Silvey 1975). Here, α is y_{max}; γ is a measure of how quickly y approaches y_{max} as x increases, and $\log(2 + \beta)/\log \gamma$ is the x-value at half maximal saturation. Normal errors of y-values were assumed, so that the fitting method was the same as least squares fitting; this was carried out using a standard computer package to minimize, iteratively, the sum of squared distances from the plotted points to the fitted curve. The identity of pairs of fitted curves was tested by means of the Asymptotic Likelihood Ratio Test (Silvey 1975) in terms of the statistic χ^2, where

$$\chi^2 = (\text{size of pooled sample}) \times \log\left(\frac{\text{RSS pooled curve}}{\text{RSS curve 1} + \text{RSS curve 2}}\right)$$

and

RSS = residual sum of squares i.e. sum of squares of distances of points from fitted curve.

Significant difference at the 95% level between the two curves requires that $\chi^2 > 7.8$.

Results

Partially purified adult human AChR showed specific activities ranging from 0.3 to 0.8 nmol α-bungarotoxin-binding sites/mg protein, the corresponding figure for foetal human AChR being 0.10 nmol/mg.

Serum from each of 3 myasthenic patients was assayed for anti-AChR antibodies by using both purified adult human AChR and purified foetal human AChR in the radioimmunoassay. In each case increasing concentrations of antigen were tested leading to a saturation curve from which antibody titre and affinity were determined. Preliminary experiments showed that unpurified AChR extracts in which receptor concentrations were frequently below 0.5 pmol/ml, were less suitable for these purposes in that concentrating the antigen, necessary in order to obtain

TABLE 1

COMPARISON OF BINDING DATA FOR MYASTHENIC SERA AND IgG WITH DIFFERENT HUMAN AChR PREPARATIONS

Data are taken from: 3 independent series of assays using 2 different foetal AChR preparations; 3 independent series of asays using 3 different adult SVD (severe vascular disease) AChR preparations; 1 series of assays using 1 adult RTA (road traffic accident) AChR preparation. Kd values are referred to as app. Kd (apparent Kd) in that they represent average values for each sample of polyclonal antisera tested.

Antigen	Patient 1				Patient 2							
	Serum				Serum				IgG			
	n [a]	Titre [b]	Kd (nM)	χ^2 [c]	n	Titre	Kd (nM)	χ^2	n	Titre	Kd (nM)	χ^2
Foetal AChR	18	122	0.30	1.30	18	210	0.34	4.05	18	149	0.39	3.10
Adult AChR (SVD)	12	124	0.30		18	192	0.31		18	128	0.32	
Adult AChR (RTA)												

Antigen	Patient 3							
	Serum				IgG			
	n	Titre	Kd (nM)	χ^2	n	Titre	Kd (nM)	χ^2
Foetal AChR	18	1416	0.32	3.57	17	815	0.25	1.99
Adult AChR (SVD)	16	1383	0.31		18	828	0.25	0.17
Adult AChR (RTA)					6	837	0.26	

[a] Number of points on binding curve.

[b] Anti-AChR antibody titre $\times 10^{-10}$ M.

χ^2 is a measure of the similarity of pairs of binding curves (see Materials and Methods). Significant difference at the 95% level between the two curves requires that $\chi^2 > 7.8$.

complete saturation, led to a greater scatter of points. The experiments were repeated for 2 of the 3 myasthenic patients by using IgG purified from their serum instead of serum itself.

Parameters obtained from the various saturation curves are shown in Table 1 in which it can be seen that the values of anti-AChR antibody titre and Kd for a given serum sample are very similar irrespective of whether purified adult human AChR or

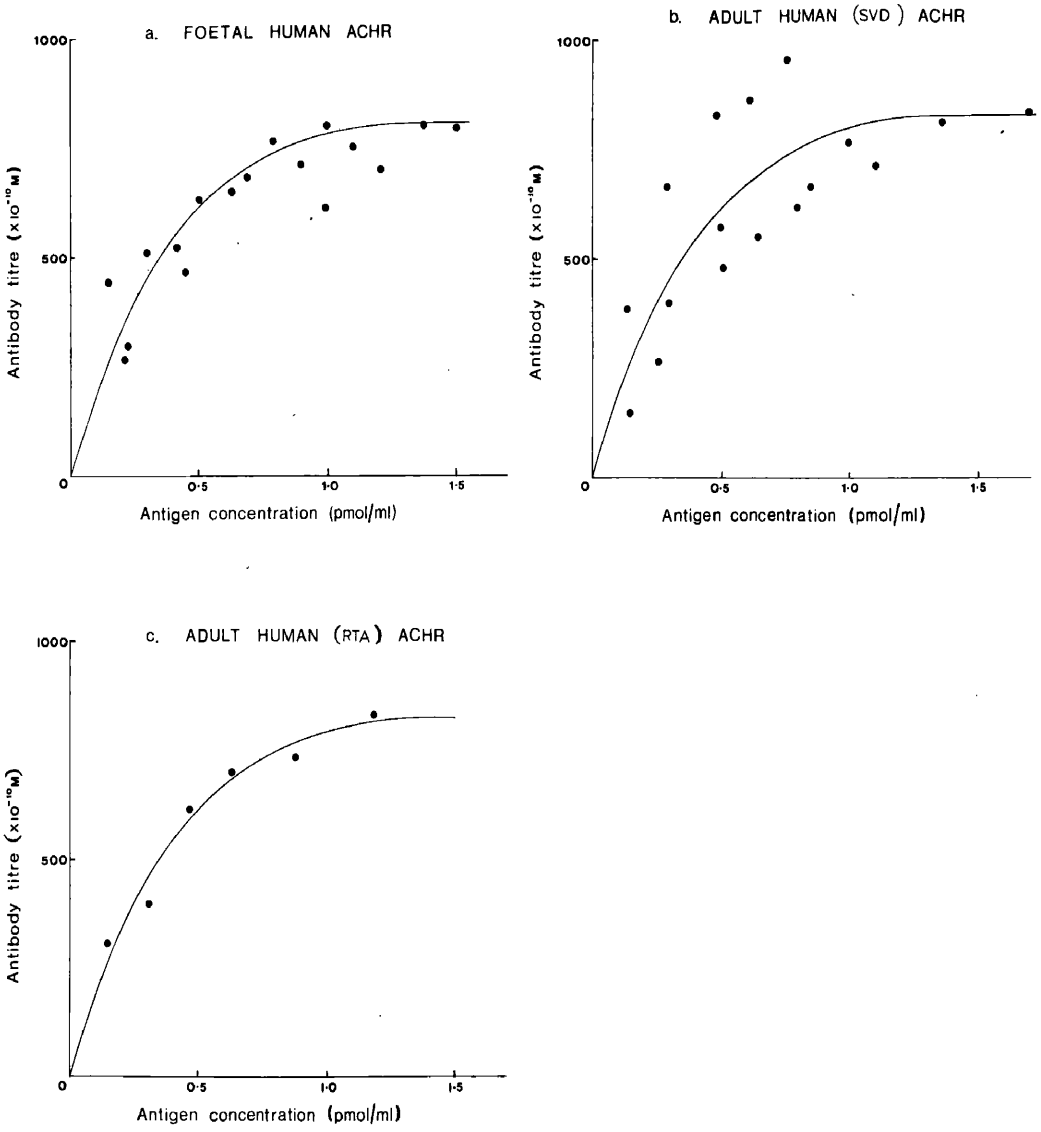

Fig. 1. Representative saturation curves for the binding of purified AChR preparations with anti-(AChR) antibodies in myasthenic IgG from patient 3 (see Table 1). An appropriate dilution of antisera was assayed with increasing concentrations of purified antigen, as described in the Materials and Methods sections. Antigens were: (*a*) foetal human AChR; (*b*) adult AChR from patients with SVD (severe vascular disease), and (*c*) adult AChR from RTA (road traffic accident) victim.

Each point is the mean of duplicate determinations. The values are taken from 2 independent assays using 2 different foetal AChR preparations (*a*); 3 independent assays using 3 different adult (SVD) AChR preparations (*b*); 1 assay using 1 adult (RTA) AChR preparation (*c*).

purified foetal human AChR is used as the antigen. The similarity of pairs of fitted curves is reflected in the χ^2 values which in no case exceed 4.10 (see Methods section).

As a check on the validity of using commonly amputated ischemic muscle as a source of normal junctional AChR, IgG from patient 3 was additionally assayed by using AChR purified from normal adult muscle (amputated followed a road traffic accident). No significant difference was found between the fitted saturation curve so obtained and that obtained with AChR from ischemic muscle.

Data for IgG from patient 3, representative of all the results in Table 1, are shown in Fig. 1.

Discussion

Preferential interaction of myasthenic IgG with denervated, as opposed to normal rat muscle AChR was first reported by Almon and Appel (1975) who demonstrated that such IgG inhibited binding of α-bungarotoxin to AChR from denervated but not normal muscle. These workers also used radioimmunoassay to show precipitation of denervated rat muscle AChR by myasthenic IgG; normal muscle AChR was not so precipitated. Weinberg and Hall (1979) subsequently extended this latter approach by the radioimmunoassay of anti-AChR antibody titres in the sera of 10 myasthenic patients by using, as antigen, either extrajunctional AChR from denervated rat muscle or junctional AChR from normal rat muscle. Titres from a given serum sample were found to differ depending on the antigen; ratios of anti-extrajunctional AChR : anti-junctional AChR antibodies ranging from 1.0 to 2.4. These findings were interpreted in terms of the presence of antigenic sites unique to extrajunctional receptor and recognized by myasthenic sera and suggested that such sera might also preferentially interact with embryonic AChR; itself extrajunctional. Indeed this was investigated in the case of 1 myasthenic serum sample which showed the same antibody titre whether assayed by using, as antigen, either AChR from denervated adult rat or that from 18-day-old rat embryos (Weinberg and Hall 1979).

Anti-rat AChR antibodies, whether anti-extrajunctional AChR or anti-normal AChR, usually comprise only a small proportion of the total anti-human AChR antibody population (Harrison et al. 1981) and the above results cannot necessarily be extrapolated to the corresponding human AChR types.

Vincent and Newsom-Davis (1979) compared myasthenic anti-AChR antibody titres obtained by using either "normal" or "denervated" adult human AChR as antigens and found essentially no differences between the two sets of titres. In these studies "normal" AChR and "denervated" AChR preparations were obtained from muscles of legs amputated from patients having respectively either arteriosclerotic ischemia or diabetes with ischemia and peripheral neuropathy. The same authors (1982) subsequently made further comparative studies in which "denervated" AChR was obtained as previously but "normal" AChR was derived post mortem from a patient dying of cerebral disease. In contrast to the earlier results, anti-"normal" AChR antibody titres were found to be variable but generally slightly lower than

anti-"denervated" AChR antibody titres and the authors suggested that the previously observed equality of titres might result from partial denervation of the ischemic "normal" muscle used in the earlier work. Lefvert (1981, 1982) has also similarly compared "denervated" AChR, again obtained from patients with diabetes mellitus and advanced neuropathy, with "normal" AChR, prepared in this case from muscle amputated from patients with malignant tumours of the bone. Some 40% of the myasthenic sera did not react with "normal" AChR but for those that did, anti-AChR antibody titres were found to be approximately 50% lower than the corresponding anti-"denervated" AChR antibody titres.

There is accordingly some evidence of a preferential interaction between myasthenic antibodies and extrajunctional, rather than junctional human AChR and the present work was designed to examine whether this preference extends to foetal human AChR. The results (Table 1) show no significant differences between antibody — "normal" adult AChR and antibody — foetal AChR binding curves for any of the myasthenic sera studied, indicating identity not only of titre but also of affinity for antibodies directed against the two receptor types. Like those of Vincent and Newsom-Davis (1979) our comparisons were largely made by using "normal" AChR obtained from muscle amputated from patients with severe vascular disease. However, a direct comparison of binding of antibodies to this AChR with that to AChR obtained from a healthy adult who had suffered a road traffic accident showed virtually identical saturation curves (Fig. 1). It appears therefore that while some ischemic muscle amputated from vascular disease patients may be partially denervated, that used in our experiments can be regarded as providing antigenically normal adult human AChR with regard to its interaction with myasthenic serum.

Our data therefore do not support the idea that foetal human AChR carries antigenic sites that are recognized by myasthenic sera and are not present on normal adult junctional AChR, although it is, of course, conceivable that such sites could have been differentially lost during the purification of foetal AChR or that the numbers of corresponding antibodies in the sera tested are very low. Vincent and Newsom-Davis (1982) comment, without data, that myasthenic sera reacted similarly with AChR from either denervated muscle or from cultured foetal human muscle; an observation which implies that anti-foetal AChR antibody titres are, like those against denervated AChR, higher than anti-normal AChR. This is in apparent contrast to our own findings although it is worth noting that the differences between anti-denervated AChR and anti-normal AChR antibody titres observed by Vincent and Newsom-Davis (1982) are not great and, moreover, that their foetal AChR is not directly comparable with ours. Our foetal AChR was obtained from prostaglandin-induced abortions, the foetuses being aged between 14 and 22 weeks, and it is certainly possible that AChR obtained from younger or older foetuses might be antigenically different.

Serum from different myasthenic patients vary in their specificities and, in particular, in their ability to distinguish between junctional and extrajunctional rat AChR (Harrison et al. 1981). However, sera from patients No. 1, 2 and 3 used in these studies had anti-junctional rat AChR: anti-extrajunctional rat AChR antibody titres of 0.29, 1.00 and 0.16, respectively, showing that differential interactions with

rat antigens are not necessarily reflected in the case of human receptors. It appears therefore, that the idea of a foetal auto-immunogen in myasthenia gravis, conceived on the basis of experiments with rat AChR (Weinberg and Hall 1979), should be further tested with more extensive studies using more myasthenic sera, and if possible a wider range of foetal receptors.

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